Assessing the role of native plant growth-promoting rhizobacteria (PGPR) isolated from Cameroon soil as bio-inoculant in improving plant growth

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Gylaine Vanissa Tchuisseu Tchakounté, M.Sc.

Präsidentin der Humboldt-Universität zu Berlin Prof. Dr.-Ing. Dr. Sabine Kunst

Dekan der Lebenswissenschaftlichen Fakultät der Humboldt-Universität zu Berlin
Prof. Dr. Bernhard Grimm

Gutachter/innen:

1. Prof. Dr. Eckhard George
2. PD Dr. Silke Ruppel
3. Prof. Dr. hab. Katarzyna Hrynkiewicz

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Dedication

To Paoni Prunelle Njima Kimbadi

Elisheva Prielle Tchakounté Defeukou

and Michelle-Archange Precious Téné Defeukou

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Abbreviations

AAS	Atomic Absorption Spectrophotometry
ABA	Abscisic Acid
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BLAST	Basic Local Alignment Search Tool
BNF	Biological Nitrogen Fixation
CAS	Chrome Azurol S
CRP	Cameroonian Rock Phosphate
DNA	Deoxyribonucleic Acid
DGGE	Denaturing Gradient Gel Electrophoresis
EDTA	Ethylene Diamine Tetra Acetic Acid
FIA	Flow Injection Analysis
FISH	Fluorescent *in situ* Hybridization
GA	Gibberelins
GLASOD	Global Land Assessment of Soil Degradation
GPD	Gross Domestic Product
IAA	Indole Acetic Acid
KOH	Postassium Hydroxyde
N	Nitrogen
NaCl	Sodium Chloride
NaOCl	Sodium Hypochlorite
NARH	Nucleic Acid Reassociation and Hybridization
NBRIP	National Botanical Research Institute's Phosphate growth medium
NCBI	National Center for Biotechnology Information
OD	Optical Density
P	Phosphorus
PCR	Polymerase Chain Reaction
PGP	Plant Growth-Promoting
PGPR	Plant Growth-Promoting Rhizobacteria
pH	potential Hydrogen
PSB	Phosphate Solubilizing Bacteria
rDNA	ribosomal Deoxyribonucleic Acid
RDP	Ribosomal Database Project
RISA	Ribosomal Intergenic Spacer Analysis
RP	Rock phosphate
rRNA	ribosomal Ribonucleic Acid
SI	Solubilization Index
SSCP	Single Strand Conformation Polymorphism
TCA	Trichloroacetic Acid
TCP	Tricalcium Phosphate
TGGE	Temperature Gradient Gel Electrophoresis
T-RFLP	Terminal Restriction Fragment Length Polymorphism

Summary

Nutrient deficiencies in soil, mainly in phosphorus (P) and nitrogen (N), coupled to salinity and the impoverishment of agricultural soils, are a severe problem for agricultural production worldwide. Therefore, there is an urgent need for research and development of more suitable agricultural practices in order to reduce unfavorable conditions, and if possible, to restore the fertility of cultivated lands. The use of rhizobacteria, which promote plant growth (PGPR), can prove useful in developing strategies to facilitate plant growth under normal as well as under abiotic stress conditions. These bacteria offer benefits to plant hosts by promoting the uptake of soil minerals and protecting plants from environmental stresses. The thesis evaluates the role of native PGPR associated with maize as potential bio-inoculants for plants growth in Cameroon. We hypothesized that native bacterial communities from Cameroon include a high potential of bacteria helping the plant cope with unfavorable conditions. Here, we provide for the first time a comprehensive phylogenetic affiliation of cultivable bacterial communities associated with maize rhizosphere grown in Cameroon in relationship to their potential plant growth-promoting abilities.

Native bacteria from Cameroon maize rhizosphere soil were isolated. Cultivated bacteria were screened for traits particularly relevant for Cameroon low-fertility soil conditions, such as their abilities to tolerate high concentrations of salt, and their plant growth-promoting potential. Genetic and functional diversity of the isolates was characterized according to their phylogenetic affiliation using molecular/bioinformatics tools. A total of 143 bacteria were identified and assigned to 3 phyla (*Actinobacteria*, *Firmicutes* and *Proteobacteria*), 13 families and 20 genera. Based on their *in vitro* characterization, 88.1% were salt tolerant at 2% NaCl, but only 16.8% could tolerate 8% NaCl, 50.4% solubilized phosphate, 10.5% possessed the *nifH* gene, and 19.6% produced siderophores. From the two most dominant genera, *Arthrobacter* and *Bacillus*, identified in this work, three strains of *Arthrobacter* and three of *Bacillus*, bearing different plant growth-promoting (PGP) traits, were selected for further studies. First, we demonstrated that strains with more identified PGP traits, significantly increased maize seedling growth in an *in vitro* germination assay. Then, we evaluated the bacteria's ability to cope with phosphorus (P) and salt stress at the same time. Selected *Arthrobacter* and *Bacillus* strains were further *in vitro* tested for their phosphate solubilizing and motility abilities under salt stress condition. Additionally, they were also evaluated for their capacities to produce different types of phytohormones. All the six selected bacterial strains mobilized P from different phosphate sources in shaking culture under both non-saline and saline conditions. They also showed the ability to produce indole acetic acid (IAA), abscisic

acid (ABA), gibberellins (GAs) and cytokinins (CKs), known to play a vital role in the stimulation of plant growth and defense response against stresses.

Further, we hypothesized that selected *Arthrobacter* and *Bacillus* strains will be helpful to promote tomato plant growth. In order to verify that the plant stimulating effects of selected bacterial strains were not specific to their host plant, the ability of these bacteria to promote tomato growth under combined P and salt stress condition was investigated in greenhouse experiments. Finally, with aim to prove the bacterial strain - plant cultivar dependent interaction between selected PGPR and maize cultivars, composites and hybrids cultivars, we evaluated the response of four indigenous maize cultivars (two composites and two hybrids) widely grown in Cameroon to the selected *Arthrobacter* and *Bacillus* strains under combined P and salt stress condition. Greenhouse experiment results showed the capacity of the six *Arthrobacter* and *Bacillus* strains to enhance tomato and maize plant growth in P-deficient and salt-affected soils. However, some strains with higher plant growth-promoting potential induced higher and more stable growth effects. Interestingly, the capacity of bacterial plant growth- stimulating in greenhouse was higher in composite than in hybrid cultivars although the growth performance of hybrid cultivars with or without bacterial treatment was still better compared to the composite cultivars. The results of this thesis provide valuable information for prospective production of effective bio-fertilizers based on native PGPR with multiple PGP traits, applicable even under combined P-deficiency and salt stress and support the fact that co-selecting of a proper plant cultivar along with the highly efficient appropriate PGPR strain is advisable for improvement of plant growth under a given condition.

Zusammenfassung

Der Mangel an Nährstoffen im Boden, hauptsächlich an Phosphor (P) und Stickstoff (N), verbunden mit einem hohen Salzgehalt und der generellen Verarmung landwirtschaftlicher Böden , sind ein ernstes Problem für die landwirtschaftliche Produktion weltweit. Daher besteht ein dringender Bedarf an Forschung und Entwicklung geeigneter landwirtschaftlicher Praktiken, um ungünstige Bodenbedingungen zu verringern und wenn möglich die Fruchtbarkeit von Kulturland wiederherzustellen. Die Verwendung von Rhizobakterien, die das Pflanzenwachstum (PGPR) fördern, kann sich bei der Entwicklung von Strategien zur Erleichterung des Pflanzenwachstums unter normalen Wachstumsbedingungen sowie unter abiotischen Stress als nützlich erweisen. Diese Bakterien bieten ihren pflanzlichen Wirten Vorteile, indem sie die Aufnahme von Bodenmineralien fördern und Pflanzen vor schädlichen Umwelteinflüssen schützen. Die vorliegende Arbeit bewertet die Rolle von in Kamerun natürlich vorkommenden PGPR an Mais und untersucht deren Potenzial als Bioimpfstoffe zur Steigerung des Pflanzenwachstums in Kamerun. Wir prüfen die Hypothese, dass einheimische Bakteriengemeinschaften aus Kamerun einen hohen Anteil an Bakterien aufweisen, deren Eigenschaften Kulturpflanzen helfen, mit ungünstigen Bedingungen umzugehen. In der vorliegenden Arbeit wurden dazu Bakteriengemeinschaften der Rhizosphäre von in Kamerun angebautem Mais isoliert und untersucht. Zum ersten Mal erfolgte eine umfassende phylogenetische Zuordnung aller kultivierbaren Bakterien, auf Grundlage ihrer potenziellen Fähigkeiten zur Förderung des Pflanzenwachstums.

Die kultivierbaren Bakterien wurden auf Merkmale untersucht, die besonders für Bodenbedingungen mit geringer Fruchtbarkeit in Kamerun relevant sind. So waren z. B. ihre Fähigkeit, hohe Salzkonzentrationen zu tolerieren, und ihr Potenzial Phosphat zu lösen und für die Pflanze zur Verfügung zu stellen, wichtig.

Unter Verwendung molekularer Techniken und bioinformatischer Auswertungsprogramme wurden die Isolate phylogenetisch zugeordnet und funktionell charakterisiert. Insgesamt konnten 143 Bakterien identifiziert und diese 3 Phyla (Actinobacteria, Firmicutes und Proteobacteria), 13 Familien und 20 Gattungen zugeordnet werden. Basierend auf ihrer *in-vitro*-Charakterisierung tolerieren 88,1% der Bakterien 2% NaCl, aber nur 16,8% eine Konzentration von 8% NaCl. Insgesamt lösen 50,4% der Bakterien Phosphat, 10,5% besitzen das *nifH*-Gen und 19,6% produzieren Siderophore. Aus den beiden, in dieser Arbeit identifizierten, dominierenden Gattungen *Arthrobacter* und *Bacillus*, , wurden jeweils drei Stämme mit unterschiedlichen Merkmalen zur Förderung des Pflanzenwachstums (PGP) für weitere Studien ausgewählt. Es zeigte sich, dass Stämme mit einer höheren Anzahl an identifizierten PGP-Merkmalen das Wachstum von Maiskeimlingen im *in-vitro*-Keimungstest,

im Vergleich zu denen mit einer geringeren Anzahl, signifikant erhöhten. Eine entscheidende Fragestellung dieser Arbeit war, inwiefern die isolierten Rhizobakterien fähig sind, gleichzeitig mit Phosphor (P) und Salzstress umzugehen. Deshalb wurden die ausgewählten *Arthrobacter*- und *Bacillus*-Stämme weiter *in vitro* auf ihre Phosphatlöslichkeits- und Motilitätsfähigkeiten unter Salzstressbedingungen getestet. Alle ausgewählten Bakterienstämme mobilisierten P aus verschiedenen Phosphatquellen in Schüttelkultur sowohl unter nicht-salzhaltigen als auch unter salzhaltigen Bedingungen.

Ferner stellten wir die Hypothese auf, dass ausgewählte *Arthrobacter*- und *Bacillus*-Stämme hilfreich sein werden, um das Pflanzenwachstum bei anderen Pflanzenarten als Mais zu fördern. Dabei kann die Fähigkeit zur Produktion von Phytohormonen relevant sein. Phytohormone sind bekannt, dass sie eine wichtige Rolle bei der Stimulierung des Pflanzenwachstums und den Abwehrreaktionen der Pflanze spielen. Deshalb wurden die Bakterien auch auf ihre Fähigkeit zur Herstellung verschiedener Arten von Phytohormonen untersucht. So zeigten alle Bakterien die Fähigkeit, Indolessigsäure (IAA), Abscisinsäure (ABA), Gibberellinsäure (GAs) und Cytokinine (CKs) zu produzieren. Um zu verifizieren, dass die pflanzenstimulierenden Wirkungen der Bakterien unter P- und Salzstressbedingungen nicht wirtsspezifisch sind, wurden neben Maispflanzen auch Tomatenpflanzen inokuliert. Inwiefern jedoch auch die Wahl der Pflanzen Sorte einer Art Einfluss auf die Wechselwirkung mit einem Bakterienstamm hat, untersuchten wir an vier in Kamerun weit verbreiteten einheimischen Maissorten (zwei Kompositen und zwei Hybriden). Die vier Maissorten wurden unter kombinierten P- und Salzstressbedingungen kultiviert und mit je einem der drei ausgewählte *Arthrobacter* bzw. *Bacillus* Stämme inokuliert. Alle getesteten Stämme zeigten die Fähigkeit das Wachstum von Tomaten- und Maispflanzen in Böden mit P-Mangel und Salzbelastung zu fördern. Stämme mit höherer Anzahl verschiedener pflanzenwachstumsfördernder Fähigkeiten induzierten höhere und stabilere Wachstumseffekte. Interessanterweise erwies sich die Fähigkeit zur Stimulierung des Wachstums bei Kompositsorten höher als bei Hybridsorten, obwohl die Wachstumsleistung von Hybridsorten mit oder ohne Bakterienbehandlung im Vergleich zu Kompositsorten immer noch besser war. Die Ergebnisse dieser Arbeit liefern zu einem wertvolle Informationen für die zukünftige Herstellung wirksamer Bioinokula auf der Basis von nativen PGPR mit multiplen PGP-Merkmalen, und unterstreichen zudem die nötige Relevanz einer geeigneten, situationsangepassten Bakterien-Pflanzensorten Kombination zur Erbringung bestmöglicher Effekte.

CHAPTER 1: GENERAL INTRODUCTION

1.1. Importance, constraints, and strategies to improve agricultural production in Cameroon

1.1.1. Importance of agriculture

Agriculture contributes to a major share of national income and export earnings in many developing countries, while ensuring food security and employment (Singh et al., 2015). There are about 6.38 billion hectares of earth surface, currently occupied by ca. 7.41 billion people, of which 1.3 billion are directly dependent on agriculture (Gouda et al., 2018). With the alarmingly growing human population, expected to reach 8-9 billion by 2030, the number of people directly dependent on agriculture and the agricultural production are projected to significantly increase, too (Egamberdieva et al., 2017a). Thus, for the next 40 years, an increase of 60% of the agricultural production is conceivably required to meet the worldwide food demands (Berger et al., 2018).

In many developing countries, agriculture contributes to a major share of national income and export earnings, while ensuring food security and employment (Singh et al., 2015). Cameroon, like many other developing countries, is still dependent on agriculture for food and livelihood with the exploitation of natural resources remaining the driving force for the country's economic growth and development. Farming is a vital sector involving 80% of the country's poor and contributing about 45% of the Gross Domestic Product (GPD) (Tabe-Ojong and Molua, 2017). In addition, agricultural sector is responsible for providing food security to both the Cameroonian rural and urban populations via local production. Small-scale farmers make up the majority of food producers. The most important food crops produced include corn and tomato amongst others.

Maize production

Maize (*Zea mays* L.), also called corn, belongs to the tribe *Maydae*, family *Poaceae*, in the order Poales. It originates from Mexico and Central America. Maize is a tall, monoecious annual grass with overlapping sheaths and broad conspicuously distichous blades (Fig. 1). It possesses somatic chromosome number of 20, a genome size of 2.3 gigabase and more than 32,000 genes (Hossain et al., 2016). It is a wind-pollinated plant and both self- and cross-pollination is usually possible. Cross-pollination represents about 95%, whereas self-pollination represents 5 % of the process (Tandzi, 2014). An average temperature of 20-24°C is optimum for growth, and the temperature should not sink below 14°C at night, whereas the minimum for germination is 8°C.

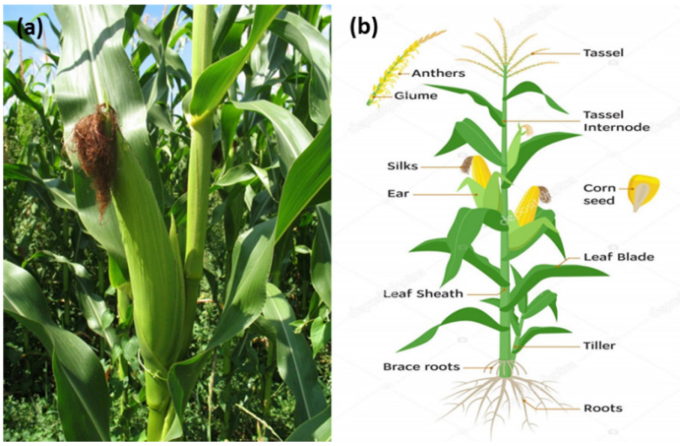


Figure 1. (a) Maize plant in a farm, in Cameroon (photo: G.V. Tchuisseu); (b) maize plant diagram infographic element (source: https://www.vectorstock.com/royalty-free-vector/maize-plant-diagram-infographic-elements-with-the-vector-23593206 accessed the 25.06.2019).

Maize is the most versatile crop, grown in more than 166 countries across the globe including tropical, subtropical, and temperate regions, from sea level up to 3,000 m altitude. In 2017, the total world production of maize reached 1.13 billion tons, with the United States of America, China, and Brazil harvesting 33%, 23%, and 8% of the total production, respectively (FAOSTAT, 2017). The whole continent of Africa is known to contribute only 7% of the global production (Ranum et al., 2014).

Maize has emerged as a crop of global importance due to its multiple end uses as a human food and livestock feed and serves as an important component for varied industrial products. Besides, maize serves as a model organism for biological research worldwide (Hossain et al., 2016). Furthermore, it is used as basic raw material for the production of starch, oil, beverages, food sweeteners and, more recently, fuel. A major portion of maize produced worldwide is used for human and animal consumption (Hossain et al., 2016). In sub-Saharan Africa, for instance, maize is a staple food for an estimated 50% of the population and provides 50% of the basic calories. Indeed, maize is an important source of carbohydrate, protein, iron, vitamin B, and minerals. Maize grains have great nutritional value as they contain 72% starch, 10% protein, 4.8% oil, 8.5% fiber, 3.0% sugar and 1.7% ash (Ranum et al., 2014).

In Cameroon, maize cultivation is mainly practiced by small-scale farmers, yielding about 2.1 tons/ha for a total production of about 2246241 tons in 2017, according to FAO (FAOSTAT, 2017). Apart from its important human consumption, by around 12 million Cameroonians, maize is the first ingredient in the manufacture of cattle feed. Moreover, it is widely utilized in aviculture, accounting for 65% of the input for manufacturing poultry

feed. It annually contributes to the GDP with about CFAF 5.6 billion, i.e., about Euros 8.6 million (Jacob et al., 2017).

Tomato production

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown vegetables in the world. It originates from western South America, but its cultivation is thought to have occurred in Central America (Kimura and Sinha, 2008). It belongs to the extremely large family *Solanaceae*, which has more than 90 genera, including many commercially important plants, such as potato, eggplant, peppers, tobacco, and petunias. Tomato is a perennial, diploid and self-pollinating plant, growing in fields or in greenhouses depending on the outdoor conditions. Most of greenhouse-grown tomato plants are cultivated in hydroponic systems, where plants can optimally be supplied with mineral, nutrients and water. In 2017, the global production of tomato reached 182,301,395 tons, with China, India and USA being the most important producing countries.

Tomato fruits are a good source of vitamin C, folate, pro-vitamin A, and potassium. One of its micronutrients, lycopene, a carotenoid that gives the red color to the fruit, helps to prevent some major diseases, such as some types of cancer and heart diseases (Rao and Agarwal, 2000). As an important ingredient in most diets, tomato also greatly improves the dietary habits. Besides its nutritional and commercial value, tomato is now considered a model plant (Kimura and Sinha, 2008) and it has been intensively used in research.

In Cameroon tomato is cultivated in open field and its annual production is estimated at 877,937 tons, with a yield level of 12,286 kg ha^{-1} (Tabe-Ojong and Molua, 2017). Tomato production provides both income and employment to small-holder and medium scale commercial farmers, as part of the harvest is used for domestic consumption and the other part is sold locally and/or exported to other Central African countries.

1.1.2. Constraints of agricultural production

Worldwide, but particularly in developing countries, food security is threatened by both, the growing population and the climate change. As a result of increasing urbanization and industrialization, threats to the environment have increased, leading to the shrinkage of agricultural land on one hand and causing significant declines in crop growth on the other hand (Egamberdieva et al., 2017a). The Global Land Assessment of Soil Degradation (GLASOD) mapping system estimated that 8.7 billion hectares of agricultural land, pasture, and forest and 2 billion hectares of woodland have been degraded since 1950. The aforementioned mapping system also noticed that 3.5% of the total cultivated lands have been degraded severely, while 10% were moderately ruined, and 9% were lightly destroyed.

Another striking outcome of this study revealed that the degradation of cropland appears to be most extensive in Africa, affecting 65% of cropland area, compared with 51% in Latin America and 38% in Asia (Mishra et al., 2017). Furthermore, indiscriminate use of fertilizers, particularly nitrogen (N) and phosphorus (P) fertilizers, has led to substantial pollution of soil by reducing pH and exchangeable bases; thus, making these nutrients unavailable to crops and leading to loss of productivity (Gouda et al., 2018). In addition to land degradation due to numerous anthropogenic activities, low nutritional status and physical and biological properties of soil, incidence of pests and diseases, fluctuating climatic factors and abiotic stresses are the interlinked contributing factors for reduced agricultural productivity (Gopalakrishnan et al., 2014). Among environmental stresses, soil acidity, salinity, and deficiency in essential plant nutrients, mainly P and N, are among the most important factors restricting crops production worldwide and in Cameroon especially.

Phosphorus deficiency

After nitrogen (N), phosphorus (P) is the second most important nutrient for agricultural crop production and it makes up about 0.2% of a plant's dry weight (Alori et al., 2017). P is abundant in soils in both, inorganic and organic forms. However, it is a major factor limiting plant growth. Inorganic P occurs in soil, mostly in insoluble mineral complexes, some of them appearing after frequent application of chemical fertilizers. Organic P in soil is found in plant remains, composts and microbial tissues, and it represents an important reservoir of immobilized P that accounts for 20–80% of P in soils (Sharma et al., 2013). On average, P content of soil is about 0.05% (w/w), but only 0.1% of this P is available for plant use, as P is in an unavailable form for root uptake (Alori et al., 2017). Such a very limited P concentration in the soil for plant utilization is the leading cause of low agricultural production for about 30-40% of the world's arable lands (Abbas et al., 2018).

P deficiency in soil is due first of all to less total P contents in the soil and then to the fixation of added P from chemical fertilizers and from other organic sources, like manures. The term P fixation is used to describe reactions that remove available phosphate from the soil solution into the soil solid phase. There are two types of reactions: (a) phosphate sorption on the surface of soil minerals and (b) phosphate precipitation by free Al^{3+} and Fe^{3+} in the soil solution (Sharma et al., 2013). The fixation of P into insoluble complexes renders these compounds inaccessible for absorption by plants. Therefore, both acidic and alkaline soils typically show a severe P-insufficiency (Suleman et al., 2018).

In Cameroon, about 85% of soils are acidic, characterized by high levels of aluminum and iron, which makes the P deficiency a major, widespread constraint. For some regions, like the Littoral Region, the situation is even more appealing, as P levels are below critical

values. Overall, the P-deficient soil have been responsible for about 67% reduction of the Cameroonian maize yields (Mapiemfu-Lamare et al., 2012). Globally, the poor fertility of acid soils is known to result from a combination of mineral toxicities (Al, Mn and Fe) and deficiency caused by leaching or decreased availability of P, Ca, Mg, and some other micronutrients, especially Mo, Zn, and B (Tandzi, 2014).

Figure 2. Phosphorus deficiency symptoms, purpled leaves, on tomato plants (photo, G.V. Tchuisseu)

Salinity

Globally, in terms of environmental stresses, saline stress is one of the most severe stress that effect not only the soil and plant growth, but also the living organisms (Numan et al., 2018). Salt stress may be defined as the osmotic forces exerted on plants when the later are growing in a salt marsh or under other excessively saline conditions (Numan et al., 2018). Measuring the electrical conductivity of the soil defines salinity of that particular land. When the extract solution of the soil has electrical conductivity of 20 mM (~2 dS m^{-1}) or more, it is considered as saline soil or salt affected soil (Numan et al., 2018). Soil salinization is caused by natural or human activities that increase the concentration of dissolved salts, predominantly sodium chloride, in the soil (Ilangumaran and Smith, 2017). Primary salinity is caused by natural processes, leading to significant salt accumulation in soil and ground water over extended periods. This results in the formation of salt lakes, salt marshes, marine sediments, and salt scalds in the landscape. Secondary salinity, by contrast, is caused by cultivation operations, such as land clearing, excessive irrigation, and inadequate drainage, Secondary salinization has degenerated vast tracts of irrigated lands to the point that they are no longer economically relevant for cultivation (Ilangumaran and Smith, 2017). More than 800 million hectares of land throughout the world are salt affected, either by salinity (397 million ha) or the associated condition of sodicity (434 million ha) (Niu et al., 2012). In Cameroon, salty soils represent a significant proportion of land available, covering about 2,000 km^2 (Kouam et al., 2017).

Effects of salinity on crop productivity are more severe in arid and semiarid regions, where these effects are further exacerbated by limited rainfall, high evapotranspiration, high temperature, poor water quality, and poor soil management practices (Niu et al., 2012). Salinity affects plant growth and yield of crops in varying degrees (Table 1). Crops such as cereals (rice and maize), forages (clover) or horticultural crops (potatoes and tomatoes) are relatively susceptible to excessive concentration of salts, either dissolved in irrigation water or present in soil (rhizosphere) solution (Paul and Lade, 2014). Salinity in the rhizosphere region adversely affects the growth of plants, as the roots cannot pull out water from salt accumulated areas. The other major problem is the high level of salt ions in water, which has deleterious effects on the plant growth. In fact, the abundant level of Na^+ and Cl^- ion in the soil causes the high osmotic potential of soil solution and deficiencies in nutrient uptake. Moreover, the high concentration of Na^+ and Cl^- in the soil effects the presence of other vital elements in the soil and can reduce ability of the plant to access and to uptake essential nutrients and minerals (Numan et al., 2018). Some plants, nonetheless, have developed a variety of mechanisms to sustain productivity even when being under salt stress environment. These mechanisms include osmotic adjustment, Na^+ and/or Cl^- exclusion, and tissue tolerance of high concentrations of Na^+ and/or Cl^- (Munns and Tester, 2008).

Table 1: Excess soil salinity causes poor and spotty stands of crops, uneven and stunted growth, and poor yields; The extent of its effects depends on the degree of salinity (Paul and Lade, 2014).

Salinity class	Conductivity of the saturation extract (dS m^{-1})	Effect on crop plants
Non-saline	0-2	Salinity effects negligible
Slightly saline	2-4	Yields of sensitive crops may be restricted
Moderately saline	4-8	Yields of many crops are restricted
Strongly saline	8-16	Only tolerant crops yield satisfactorily
Very strongly saline	>16	Only a few very tolerant crops yield satisfactorily

1.1.3. Strategies to improve agricultural production in Cameroon

Selection and breeding have always been conducted to achieve high yield and better quality of crops under stressful conditions (Niu et al., 2012). The Cameroonian government has made many attempts to improve the productivity of smallholder agriculture. This involves in particular the development of high yielding varieties, and subsidization of improved seed

varieties resistant to soil acidity and salinity. In the frame of this approach, Cameroon takes part to many international and regional actions aiming at the improvement of agricultural production. As an example, the Cameroonian public institution INRA actively participated in the project titled "fitting maize into cropping systems on acid soils of the tropics". This project was carried out within the International Cooperation with Developing Countries consortium, grouping many countries from Latin America, Africa, and Europe. It aimed at the selection of maize inbred lines, hybrids and composites varieties adapted to acid soil environments (Welcker et al., 2005). Besides the abovementioned efforts, expansion of cropping land, liming, application of organic fertilizers have also been used to manage soil acidity and soil salinity in croplands (Mapiemfu-Lamare et al., 2012). Moreover, N and P fertilizers are added in soils to address the problem of nutrient deficiency in different crops, but the access to these chemical products is very limited, mainly because they are too expensive for the farmers.

Despite all these efforts, low agricultural production and, thus, food insecurity continue to be a real problem in Cameroon. In 2014, for instance, the yields of maize and tomato in Cameroon (2.1 tons ha^{-1} and 12,286 kg ha^{-1}, respectively) were much lower than the world mean values of 4.2 tons ha^{-1} for maize and 33,988 kg ha^{-1} for tomato (FAOSTAT, 2014). Also, maize importation drastically rose, from 3,044 tons in 2013 to 19,964 tons in 2014 (i.e., an increment of ca. 556%), despite the high percentage of farmland used for maize production, estimated to be about 60% (Tandzi, 2014).

Due to the adverse ill-effects of chemical fertilizers on the environment and ecology, and because of the very restricted access to mineral fertilizers by farmers particularly in developing regions, nowadays bio-fertilizers are being projected as an essential component of organic farming not only for the improvement of agricultural production, but also for the maintenance of long-term soil fertility and sustainability. Plant growth-promoting rhizobacteria (PGPR) are considered to be one of the most important components of bio-fertilizers. Development of bacterial inoculants to reduce the use of chemical fertilizers without compromising plant yield and quality is a highly rewarding, but challenging task, which is increasingly attracting many scientists from the fields of agriculture, microbiology, and biotechnology (Vejan et al., 2016).

Application of PGPR as bio-fertilizer is economical, eco-friendly, productive, more efficient, and accessible to marginal and small farmers over chemical fertilizers (Kumari et al., 2018). Intensive research attempts are underway to improve the growth of plants, their tolerance to various abiotic stresses, and their protection against soil borne pathogens by using PGPR (Berger et al., 2018; Egamberdieva et al., 2017b). In the last decade, there has

been an increasing focus on the implementation of PGPR as a sustainable option to compensate for poor soil fertility conditions in developing countries and reports on enhancement of plants growth through PGPR are widely available (Abiala et al., 2015; Tchuisseu Tchakounte et al., 2018). In Cameroon, however, there is still paucity of information on both, the research on PGPR and their potential application for sustainable development.

1.2. Rhizosphere and plant growth-promoting rhizobacteria

1.2.1. Interaction between plant and microbes in the rhizosphere

Plants have always been in a relationship with microorganisms during their growth and development (Gouda et al., 2018). In fact, plants obtain their nutrients for growth mainly from soil, in which microbes are also competing for resources. The interactions of plants with microbes both in the soil and above ground shoot are of great importance in agricultural and natural ecosystems, since microbes are involved in different functions related to soil fertility and plant health such as nutrient cycling, organic matter formation and decomposition, and plant growth promotion (Kennedy, 1999).

In soil there is a zone of interaction between plants and microbes considered as a hot spot due to the high microbial activity (Gray and Smith, 2005). This zone is the rhizosphere and it is defined as the soil region where processes mediated by microorganisms are specifically influenced by the root system (Gray and Smith, 2005; Souza et al., 2015). Largely, three separate, but interacting components are recognized in the rhizosphere: the rhizosphere (soil), the rhizoplane, and the root itself. Of these, the rhizosphere is the zone of soil influenced by roots through the release of substrates that affect microbial activity. The rhizoplane, on the other hand, is the root surface including the strongly adhering soil particles. The root itself is considered as a component of the system because many microorganisms (like endophytes) also colonize the root tissues (Ahemad and Kibret, 2014).

In the rhizosphere, plant and microbes have a reciprocal influence. The root influences the rhizosphere through many activities. Besides providing the mechanical support and facilitating water and nutrients uptake, plant roots also synthesize, accumulate, and secrete a diverse array of compounds called root exudates. These root exudates, among them sugars, amino acids, organic acids, flavonoids, hormones, and vitamins, are a source of carbon and other different nutrients for soil occupying microorganisms (Ahemad and Kibret, 2014). On the other hand, bacteria have also a marked influence on the plant in the rhizosphere and their action with plants may be neutral, harmful, or beneficial (Souza et al., 2015; Whipps, 2001). Commensals bacteria establish an innocuous interaction with the host plants exhibiting no visible effect on the growth and the overall physiology of the host

(Bhattacharyya and Jha, 2012). Bacteria involved in negative interactions, which are referred to as phytopathogenic rhizobacteria, produce phytotoxic substances such as hydrogen cyanide, ethylene or make metals more readily available for plants by producing chelating molecules that degrade organic acids released by plants, thus negatively influencing plant growth and physiology (Bhattacharyya and Jha, 2012). The soil occupying bacteria that are beneficial for the plant, i.e., able to promote plant growth and development, are generally called plant growth-promoting rhizobacteria (PGPR).

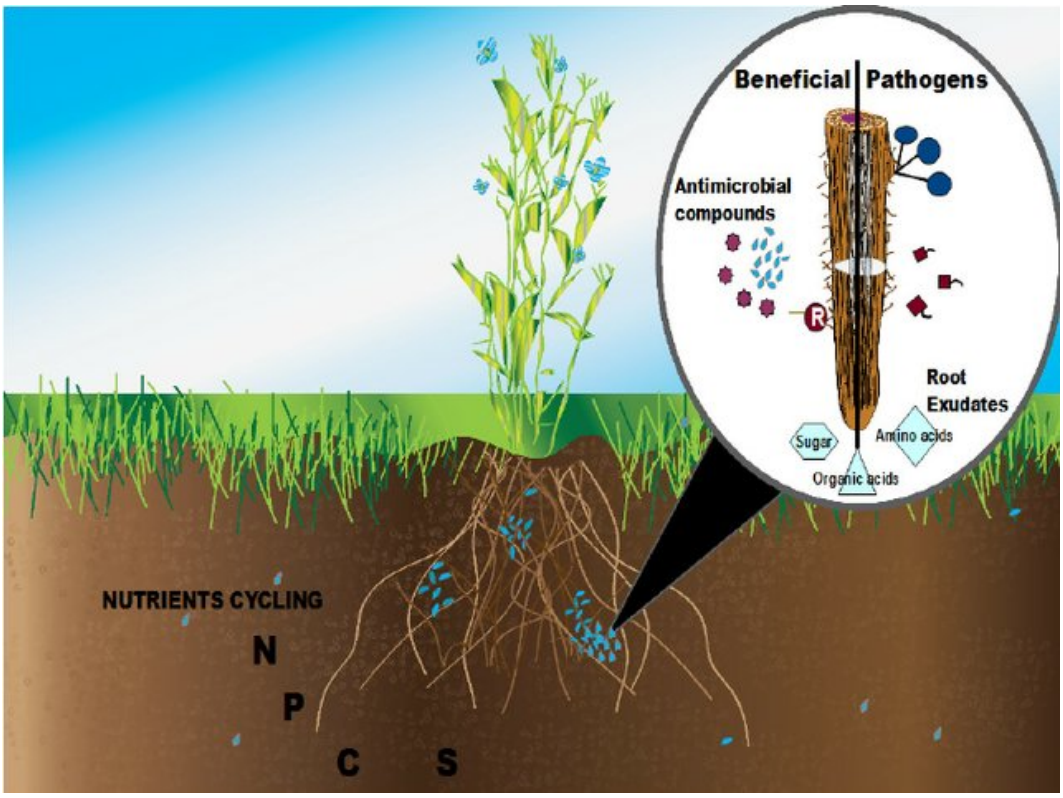


Figure 3. Examples of plant-microbe interactions in the rhizosphere: plant roots release exudates comprised of sugars, organic acids, and amino acids that may attract beneficial microbes. In exchange, the attracted microbes protect the plant against pathogens by releasing antimicrobial compounds or by increasing nutrients uptake. On the other hand, these carbon-containing compounds can also attract pathogens, which can compete for nutrients, infect the plant, and affect the rhizosphere microbial community (Rincon-Florez et al., 2013).

1.2.2. Plant growth-promoting rhizobacteria

PGPR are heterogeneous naturally-occurring soil bacteria able to benefit plants by improving their productivity and immunity (Garcia-Fraile et al., 2015). About 2 to 5% of total rhizosphere bacteria, when reintroduced in a soil containing competitive microflora by plant inoculation, exert a beneficial effect on plant growth (Antoun and Prévost, 2005). PGPR are characterized by the following inherent distinctiveness: (i) they must be proficient to colonize the root surface, (ii) they must survive, multiply and compete with other

microbiota, at least for the time needed to express their plant growth promotion/protection activities, and (iii) they must promote plant growth (Ahemad and Kibret, 2014).

The exact mechanisms by which PGPR promote plant growth are not fully understood, however PGPR are known to influence plant growth by various direct and indirect ways (Fig. 4) (Garcia-Fraile et al., 2015). PGPR can directly facilitate the growth and development of plants by increasing the availability and uptake of nutrients. Known mechanisms are such as biological N_2 fixation, solubilization of inorganic phosphate and mineralization of organic phosphate and/or other nutrients; production of siderophores and synthesis of plant growth regulators like auxin, cytokinins, gibberellins (GA), abscisic acid (ABA) and ethylene (Berger et al., 2015; Garcia-Fraile et al., 2015). Indirect stimulation involves the process through which PGPR prevent or neutralize the deleterious effects of phytopathogens and environments on plants. This include the production of repressive substances (i.e., antibiotics), the induction of the systemic resistance in plants, the creation of iron-limiting conditions through the production of siderophores that control the proliferation of phytopathogens, and the protection of plants growth actively under environmental stress (abiotic stress) (Gopalakrishnan et al., 2014; Gouda et al., 2018; Ruppel et al., 2013). A particular PGPR may affect plant growth and development by using one or combined different mechanisms; it can also use different traits for growth promotion at various times during life cycle of the plant (Beneduzi et al., 2012). Several studies are still going on to understand the diversity and importance of soil PGPR communities and their roles in betterment of agricultural sustainability.

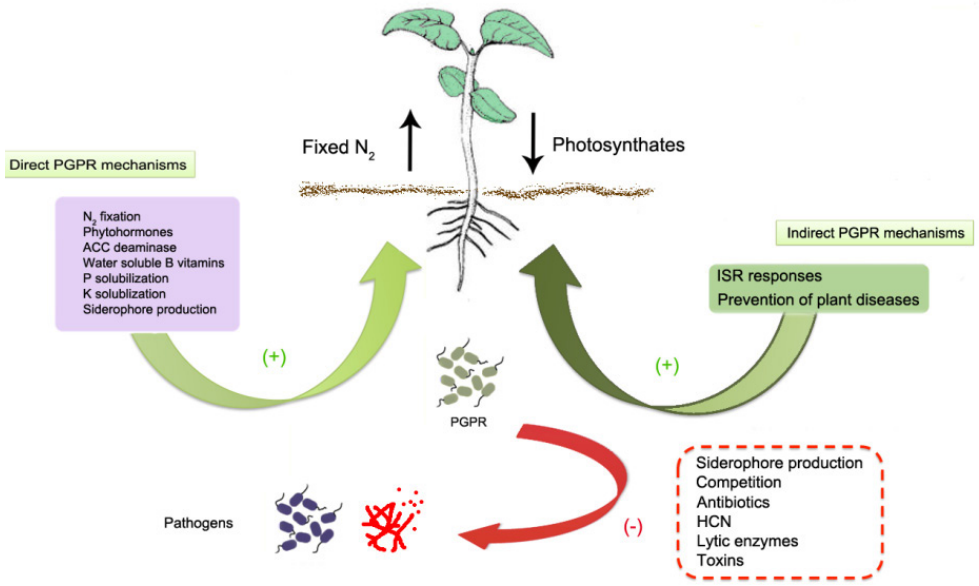


Figure 4. Direct and indirect mechanisms used by PGPR to enhance plant growth and development (Garcia-Fraile et al., 2015).

1.2.3. Role of PGPR in agriculture

The use of plant growth-promoting microorganisms in agriculture, as well as in phytoremediation, has become a very attractive technology and area of research. PGPR and their interactions with plants are exploited commercially as they have good impact in crop productivity by stimulating plant growth, increasing crop yields, being less harmful to the environment and also reducing the cost of chemical fertilizers. PGPR are used as inoculant for bio fertilization, bio stimulation, bio control and bio remediation (Bhattacharyya and Jha, 2012). The plant growth-promoting effects of PGPR have been demonstrated in different agronomic important crops such as maize, wheat, barley, peas, canola, soy, potatoes, lentils, radicchio, tomato, and cucumber in *in vitro* assays, greenhouse conditions, and in some cases, in field trials (Beneduzi et al., 2012; Berger et al., 2018; Berger et al., 2015; Fankem et al., 2014). The results observed in plant growth vary greatly. Examples include an increase in germination rates, higher biomass, better yields, higher leaf area, chlorophyll and nutrient contents, tolerance to drought, and disease resistance conferred to the plant (Lucy et al., 2004). Large numbers of PGPR strains possessing multifunctional traits have been described for their potent application in boosting plant activities in modern agriculture (Berger et al., 2015; Bhattacharyya and Jha, 2012; Etesami and Maheshwari, 2018).

Currently, the demand for microbial bio fertilizers is increasing worldwide owing to a higher degree of environmental awareness, the increasing number of laws protecting the environment, and the ever-expanding demand for ecological products (Garcia-Fraile et al., 2015). However, PGPR-inoculated crops are currently used on only a small fraction of agricultural lands worldwide. Although, the PGPR-based bio fertilizer market is increasing yearly and an increment of nearly 14% was anticipated by the end of the last decade, the use of bio fertilizers is yet not well explored in many parts of developing regions of the world (Sharma et al., 2019). Moreover, the variability in the performance of PGPR due to various environmental factors has a large impact on their effectiveness.

1.2.4. Factors affecting PGPR activities

The variability in the effectiveness of biological inoculants is of concern when used under different conditions or cropping systems. Hostile environmental conditions are deleterious for the root associated microbiota and effective functioning of the introduced PGPR inoculants (Egamberdieva et al., 2017a). Adverse conditions, including pathogens of the soil, low soil pH, and low rainfall during the growing season, can contribute to low colonization of PGPR and reduce their effect on plant growth. Agricultural practices such as fertilizer application may not seem as severe, but they also lead to large alterations in soil parameters that may affect the rhizosphere. Physical changes in soil structure after tillage,

crop rotation, and wastewater irrigation were shown to impact upon soil and root-associated bacteria (Castro-Sowinski et al., 2007). Climatic variations, soil type, nutrient, organic matter, and moisture content also affect PGPR functions. In fact, in a study with wheat and a *Pseudomonas* sp., results suggested that the less fertile the soil, the greater the plant growth stimulation by the PGPR (Freitas and Germida, 2011).

Other hard stresses like desiccation, salinity and temperature can limit the effects of PGPR on plants, resulting in poor productivity (Gouda et al., 2018). Salinity especially is considered as one of the most important environmental problem that inhibits many of the vital bacterial plant growth-promoting activities, such as nutrients uptake and phytohormones production (Paul and Lade, 2014). Activities of PGPR vary also according to the type of host plant. Plant factors that have an influence upon PGPR efficacy include plant age, plant species or even plant genotype and root exudates. It has been reported that specific strains of bacteria may promote plant growth only in certain crops. In one example, it was found that out of four *Pseudomonas* strains that promoted the growth of radish, only one was effective in promoting the growth of potato (Kloepper, 1980). PGPR effects may depend on the composition or activity of the indigenous microbial flora and other invertebrates found in the soil.

1.3. Characterization of rhizosphere microbiota

1.3.1. Overview on the techniques used for the assessment of the rhizosphere microbiota structure

The term "rhizosphere microbiota" refers to the collective microbial communities associated with the plant rhizosphere. In this thesis, the terms "microbial community" or "microorganisms" refers primarily to bacterial assortments, although viruses, algae, protozoa, and fungi are not normally excluded. The bacterial community can be studied through two approaches: structural and functional. To understand the structural approach, the groups of individuals, their species and abundance must be known (Barriuso et al., 2008). The crop associated bacterial community can be characterized using culture-based and culture-independent techniques. Culture-dependent techniques usually consist on extracting microorganisms from the system, culturing them in the laboratory and performing many morphological, biochemical and genetic tests (Barriuso et al., 2008). Although there have been some studies reporting the inefficacity of the culture-based approaches in providing comprehensive information on the composition of the bacterial communities, these techniques are the only way to isolate a given strain for further studied and potential utilization (Arruda et al., 2014).

Culture-independent or molecular-based methods for profiling environmental microbial communities include, among others, Denaturing and Temperature Gradient Gel Electrophoresis (DGGE and TGGE), Fluorescent *in situ* Hybridization (FISH), Single Strand Conformation Polymorphism (SSCP), Nucleic Acid Reassociation and Hybridization (NARH), Amplified Ribosomal DNA Restriction Analysis (ARDRA), Terminal Restriction Fragment Length Polymorphism (T-RFLP), Ribosomal Intergenic Spacer Analysis (RISA), and Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Kirk et al., 2004). Many of these approaches (e.g. DGGE, TGGE, ARDRA, RISA, ARISA, and T-RFLP) use of the genes coding for ribosomal RNAs, which are among the most conserved macromolecules in living systems (Atlas, 2005).

Analyses using ribosomal genes, especially 16S rRNA, were first performed by (Woese et al., 1990) and revealed that this molecule was an excellent molecular maker for bacterial phylogenetic studies. There are regions on the 16S rRNA that are highly conserved and others that comprise variable sequences. Comparing the differences in the base sequence of 16S rRNA gene can, therefore, provide useful information on the evolutionary changes and phylogenetic relatedness of microorganisms (Vasileiadis et al., 2012). The advantage of using 16S rRNA gene is that it can be used to identify isolated bacterial strains as well as to access the diversity of all bacteria in communities using culture independent approach (Arruda et al., 2014).

1.3.2. Microbiota diversity and ecology

Biologically, the rhizosphere microbiota is the most diverse reservoir of various microbial communities existing on the planet so far. It has been reported that one gram of rhizospheric soil may harbor up to 10 billion bacterial cells of possible thousands of different species, which thus makes the rhizosphere of any plant a hot spot of microbial biodiversity (Yadav et al., 2017). Biodiversity can be defined as the variety of different organisms in a system. It can be analyzed at different levels: The diversity on a local habitat is referred to as the alpha diversity, while the variation of species composition among habitats is the beta diversity, and the disparity in organisms compositions along a landscape of different habitats is addressed as the gamma diversity (Bergottini, 2015). Bacterial diversity can be defined in terms of taxonomic, genetic and functional diversity (Barriuso et al., 2008). As already mentioned in sections above, the rhizosphere microbiota constitutes a dynamic blend of bacteria, pathogenic and beneficial to humans, usually referred to as PGPR. PGPR strains occur in various taxonomic groups, which may be present simultaneously in a given soil (Vacheron et al., 2013). This suggests that taxonomically-contrasted PGPR strains may coexist in soil and colonize a same rhizosphere, along with all non-PGPR members of the

community (Vacheron et al., 2013). In fact, this possibility seems to be the rule rather than the exception.

PGPR populations contributing to a same type of function (i.e., nitrogen fixation, phosphate solubilization, plant development enhancement, etc.) belong to a same functional group. Functional group can be implemented when specific genes are documented. For instance, nitrogen fixers can be assessed using the *nif*H gene, which encodes the dinitrogenase subunit of the nitrogenase (Vacheron et al. 2018). It has been reported that some of the PGPR functional groups are taxonomically narrow, such as the *Pseudomonas* DAPG (2,4-Diacetylphloroglucinol) producers (Frapolli et al., 2012). In contrast, others are much more diversified, and some bacterial functional groups may also comprise both PGPR and non-PGPR strains. For instance, nitrogen fixing bacteria comprise PGPR as well as mutualistic symbionts and even a few pathogens (Vacheron et al., 2013). Many studies on biodiversity of microbes associated with crops found that microbes belonged to different phyla mainly: *Actinobacteria*, *Bacteroidetes*, *Balneolaeota*, *Basidiomycota*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria* and *Spirochaetes* (Araújo et al., 2013; Arruda et al., 2014; Pereira et al., 2011).

1.3.3. Root-associated microbiota of maize

The bacterial community associated with maize rhizosphere is one of the best studied among crop plants. In the last decades, many studies have explored the microbiota associated with maize plant based on culture-dependent or culture-independent methods or the combination of both (Abiala et al., 2015; Pereira et al., 2011; Roesch et al., 2007). The taxonomic affiliation of the bacteria associated with maize plants reveals a high dominance of *Actinobacteria*, *Firmicutes* and *Proteobaeteria* mainly α-*Proteobacteria* and β-*Proteobacteria* (Chelius and Triplett, 2001; Lopez-Reyes et al., 2015; Roesch et al., 2007). The bacterial community associated with maize harbors multiple orders: Actinomycetales, Burkholderiales, Clostridiales, Rizobiales, Rubrobacteriales and Xanthomonadales, and the bacterial genera commonly associated with maize have been identified as *Enterobacte*r, *Klebsiella*, *Pseudomonas*, *Bacillus* and *Azospirillum*. The following bacterial genera: *Arthrobacter*, *Azoarcus*, *Azorhizobium*, *Azotobacter*, *Beijerinckia*, *Burkholderia*, *Clostridium*, *Dechloromonas*, *Derxia*, *Enterobacter*, *Erwinia*, *Geobacter*, *Gluconacetobacter*, *Lysinibacillus*, *Mesorhizobium*, *Methylobacterium*, *Methylocella*, *Methylocystis*, *Methylosinus*, *Micrococus*, *Pseudomonas*, *Paenibacillus*, *Rahnella*, *Raoultella*, *Rhizobium*, *Rhodoblastus*, *Rhodovulum*, *Sinorhizobium*, *Staphylococcus*, *Stenotrophomona*s and *Xanthobacter* were also reported being associated with maize rhizosphere (Abiala et al., 2015; Cavaglieri et al., 2009; Gomes et al., 2014; Montañez et al., 2012; Pereira et al., 2011; Roesch et al., 2007). Some of these bacteria associated with the

rhizosphere of maize have been tested in many cops, resulting in positive effects on plants growth by increasing nutrient uptake, or reducing the effect of plant pathogens (Abiala et al., 2015; Majeed et al., 2015). Although the use of PGPR is relatively advanced in different agricultural systems worldwide, in Sub-Sahara, and in Cameroon particularly, little is known about PGPR associated with plants. In fact, research on PGPR associated with maize crop is required for understanding and utilizing root associated bacteria with the aim of growth promotion and sustainable maize cultivation in Cameroon.

1.4. Thesis hypotheses and objectives

1.4.1. Hypotheses

Plant-microbe interactions have an important role in agricultural production. In both, managed and natural ecosystem beneficial plant-associated bacteria play a key role in supporting and/or increasing plant health and growth (Dawwam et al., 2013). However, PGPR efficiency depends mainly on native biotic and abiotic factors. Indigenous isolates may be recommended in the selection of PGPR for inoculation of crop plants, as they are adapted in the environment; they can be more competitive than the non-indigenous bacterial flora. Taking into consideration several successful cases in which indigenous bacteria were used to enhance plant development, we hypothesized that rhizosphere of maize grown in Cameroon could represent a reservoir for selecting potential PGPR strains that are adapted to endemic soil conditions particularly the phosphorus-deficient and salt-affected soil.

It is well-known that PGPR that induce growth in one plant species do not necessarily have similar effects on other species (Batista et al., 2018). Some bacteria exert general growth-promotion effects on several plant species, whereas other bacteria show strong host-plant selectivity and colonize a single plant species or a limited variety of species (Long et al., 2008). Many studies have reported that positive PGPR effect sometimes occurs in crops inoculated with PGPR isolated from the same plant species (Lucy et al., 2004). Therefore, one of the effective strategies for selection of efficient PGPR is the consideration of the diversity of the host plants. We assumed that bacteria isolated from maize rhizosphere and selected for a wide range of PGP traits will not only promote maize plant growth, but also tomato.

Conventionally, PGPR application is used in assisting plant growth under normal and specific abiotic stress condition, e.g., phosphorus deficiency or saline stress. Plants are challenged, however, by multiple abiotic stresses at the same time. Additionally, previous studies have stated that multiple PGP traits are expected to be advantageous for plant development under multiple types of adverse conditions (Batista et al., 2018; Etesami and Maheshwari, 2018). Therefore, we expected that bacterial strains isolated from maize

rhizosphere in Cameroon will increase maize and tomato plant growth under double P and salt stress, and bacteria comprising multiple PGP traits will exhibit a higher effect in increasing plant growth.

Besides selecting efficient PGPR for the development of inoculants for agricultural production, the choice of adapted cultivars that benefit from association with these bacteria is a very important agronomical aspect that should also be considered. Indeed, early studies showed that the extent of positive effects of the bacteria on plant growth varied with the cultivar of the host plant (Arujo et al., 2013; Egamberdieva et al. 2010). Thus, we hypothesized that plant growth stimulating effects of selected *Arthrobacter* and *Bacillus* strains on composites and hybrids maize cultivars under combined P and salt stress condition will be different.

1.4.2. Objectives

The main goal of this study was to evaluate the role of native plant growth-promoting rhizobacteria (PGPR) as potential bio-inoculants for plant growth in P-deficient and salt-affected soils in Cameroon.

Specific objectives were to:

- Assess the cultivable bacterial community associated with maize grown in Cameroon and to characterize isolated bacteria based on phenotypic and genotypic attributes.
- *In vitro* test isolated bacteria for their plant growth-promoting functions and number of plant growth-promoting traits, including phosphate solubilization, salinity tolerance, nitrogen fixation, motility, and siderophores and phytohormones production.
- *In vitro* evaluate the effect of salt on the PGP activities of selected bacterial strains.
- Investigate the effect of selected bacterial strains on two plants (maize and tomato) in greenhouse experiment under different conditions of P and salt stress.

1.5. Thesis outline

Apart from this introductory chapter, Chapter 1, and the conclusion, the present doctoral thesis comprises five other chapters among which chapters three, four, and five consist of manuscripts that have respectively been published in refereed academic journals. Here below a short description of the contents of each chapter is provided.

Chapter 2 presents specifics of different procedures and detailed protocols used to attain data reported and discussed in this thesis.

Chapter 3 describes the isolation, characterization, and selection of rhizobacterial strains from maize rhizosphere to assess their community structure and their role as PGPR in maize seedlings *in vitro*. Our approach focused on using a combination of molecular / bioinformatics tools and *in vitro* studies to provide insight into the functional differences between and within different groups of isolates, necessary to carefully select beneficial indigenous isolates. This chapter is published in *Microbiological research*, 214, 47-59 and in *Data in Brief*, 19, 1410-1417.

Chapter 4 focuses on the selection procedure of PGPR strains acting on tomato plant under double stress. *In vitro* and *in vivo* experiments were combined to evaluate the effect of selected PGPR strains on growth and P uptake of tomato plants under P stress combined to different level of salt stress. This part is published in *Microorganisms*, *8*, 1844.

Chapter 5 shows the effect of selected bacterial strains on the growth of four maize cultivars under combined P and salt stresses. We use *in vivo* experiments to investigate the response of two composites and two hybrids maize varieties to bacterial inoculation. This chapter is published in *Microorganisms*, *8*, 1005.

Chapter 6 provides a general discussion on the most important outcomes of this thesis, the conclusion and presents further work that have to be done based on the results of this thesis.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1. Sterilizing solutions and glassware

Glassware was all sterilized by autoclaving with a "solid bodies" program at 121°C for 20 minutes. All solutions, except the heat sensitive ones, were also sterilized by autoclaving at 121°C for 20 minutes, but with the "liquids" program. All these sterilization processes were carried out in a Systec VX-75 autoclave (Systec GmbH Labour Technik, Wettenberg, Germany). The heat-sensitive solutions were sterilized by filter sterilization methods using a sterile filter with 0.22 µm pore size. Ethanol and sodium hypochlorite solutions for seeds sterilization were prepared using sterilized distilled water.

2.2. Soil nutrient analyses

Soil pH was determined according to Krey et al. (2011). Electrometric measurement of the H^+ ion activity in a suspension of soil in saline solutions of neutral reactivity (calcium chloride/$CaCl_2$) in a ratio of 1–2.5 was done using Schott Instruments Pro Lab 2000 (SI Analytics GmbH, Mainz, Germany). Available amounts of phosphate (P) and potassium (K) were extracted by the double lactate method. P and K were extracted with a solution of calcium lactate adjusted to pH 3.6 by hydrochloric acid. Afterwards, to determine P, flow injection analysis (FIA) was performed by means of colorimetric detection following ISO/EN/DIN 15681-1. The produced *ortho*-phosphate was treated with molybdate in acidic medium to produce phosphomolybdate, which was reduced to molybdenum blue by zinc-(II) chloride/ hydroxylamine. The color intensity was detected by absorbance measurement at 700 nm using a FIA module *ortho*-phosphate (Analysis technology HLS, Salzwedel, Germany). K was measured by atomic absorption spectrophotometry (ASS) at a wavelength of 769.9 nm using an atomic absorption spectrophotometer FS280 (Agilent Technologies, Waldbronn, Germany). Exchangeable magnesium (Mg) was determined using a $CaCl_2$ solution by AAS at 285.2 nm (AAS FS280, Agilent Technologies, Waldbronn, Germany). Total carbon and total nitrogen contents were measured using a VARIO elemental analyser (Elementar GmbH, Langenselbold, Germany) based on the DUMAS method (Mcgeehan and Naylor 1988).

2.3. Inorganic phosphate sources

Different inorganic phosphate sources were used for the phosphate solubilization test and greenhouse experiments. The inorganic phosphate sources included: tricalcium phosphate (TCP), hydroxyapatite, and five rock phosphates (RP) from different origins (Algeria, Cameroon, Mali, Mexico and Morocco). To remove their soluble P fractions, all phosphate sources were washed four times with warm water in the following cycle: 1h - 24h – 1h - 24h. They were then dried at 60°C until complete evaporation of water and homogenized before use.

Table 2: Chemical characteristic of the five inorganic rock phosphate sources used

Origin	(%)						mg kg^{-1}				
	Total P_2O_5	Available P	K	Ca	Mg	Na	Fe	Al	Mn	Zn	Cu
Algeria	13.85	Nd	0.12	Nd	0.78	1.08	0.27	0.24	Nd	97.50	5.72
Cameroon	11.15	Nd	0.04	Nd	0.08	0.006	30.60	3.34	Nd	46.2	10.2
Mali	30.00	12.98	0.06	28.19	0.13	0.23	3.84	0.80	8360	87	51
Mexico	28.00	8.87	0.22	25.94	0.22	0.36	0.44	0.58	788	103	18
Morocco	13.00	9.33	0.09	28.83	1.93	0.55	0.27	0.42	96	219	38

Table adapted from Fankem et al. (2014), Nd: not determined

2.4. Bacterial inocula preparation

2.4.1. Bacterial growth procedures

All strains used in this study were cultured in Standard Nutrient Agar I or Standard Nutrient Broth I (Carl Roth, Karlsruhe, Germany) as standard conditions, for instance, to prepare the inoculum for greenhouse experiments, biochemical tests or cultures for nucleic acid extraction purposes, and to purify bacterial isolates. When grown on the agar plates, the cultures were incubated in a stationary incubator. For growth in liquid cultures, a single pure bacterial colony for each strain was transferred into a 100 mL Erlenmeyer flask containing 50 mL of Standard Nutrient Broth and incubated, with rotatory shaking (at 180 rpm) for 24 h. The incubation temperature for both, agar plates and liquid culture varied between 28-30^{o}C.

2.4.2. Culture harvest under sterile conditions and optical density adjustment

After incubation, cultures were transferred into 50 mL sterile centrifuge tubes under a sterile Antares laminar hood (Thermo Fischer Scientific, Darmstadt, Germany). The cultures were washed three times by suspending the pellets in 0.05 M NaCl buffer solution and repeated centrifugation to remove the excess media residual before being adjusted/ to the desired optical density (OD). To ensure that the cultures were kept under sterile conditions, during harvesting the tubes were only opened in the sterile laminar hood and the cell pellets were rinsed or diluted when needed only with sterile 0.05 M NaCl buffer solution that was also only opened under the abovementioned laminar hood. In all experiments, bacterial OD was adjusted to prevent variations that might arise from changes in the sample volume. The quantification of bacterial OD in liquid culture was carried out on an Anthos htlll spectrophotometer (Anthos Mikrosysteme GmbH, Friesoythe, Germany) at 620 nm by measuring 100 µL bacteria

suspension in a 96 well plate. For all *in vitro* biochemical tests, bacteria inoculum was always prepared at OD_{620} at 0.2.

2.5. KOH test string for gram-positive or negative bacteria distinction

For distinction between gram-negative and gram-positive bacteria, the much quicker and easier-to-perform KOH test was used instead of the traditional gram-staining method. The KOH test was done following a well-established protocol (Gregersen, 1978). Concisely, a 3% KOH solution was prepared by dissolving 3g of KOH in 100 mL sterilized distilled water. Bacterial isolates were grown on Standard nutrient agar plate for 48h. One to two drops of 3% KOH solution were placed on a glass slide. A colony, or several small colonies, was picked from the surface of agar plate with an inoculation loop and stirred in KOH for about 1 min. Afterwards, the inoculation loop was raised from the drop. During that operation it was observed whether the liquid follows the loop. If the KOH solution became viscous and a tread of slime of 1.5 – 2 cm or more followed the loop, the reaction was positive and the bacteria considered as gram negative. If there was no slime, but a watery suspension that did not follow the loop, the reaction was negative and the isolate was gram-positive bacteria. The production of the slimy substance after treatment of gram-negative bacteria with KOH was probably due to the destruction of the cell wall and the liberation of the DNA, which is a very viscid compound.

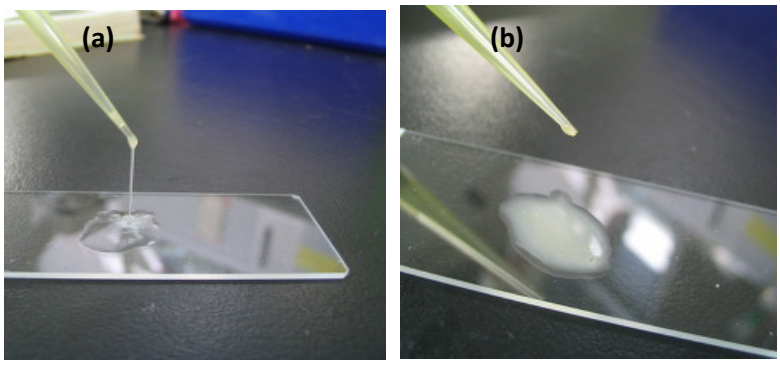


Figure 5. Bacterial identification using the KOH test: (a) gram negative bacteria showing a postive string test, and (b) gram-positive bacteria showing a negative string test

2.6. Conservation of bacterial isolates

Conservation of pure bacterial cultures for a long period was done in 50% glycerol. Bacterial strains were first cultured on standard nutrient agar for 24h by streaking a single colony to cover the entire plate. 50% glycerol prepared by mixing 50 mL of glycerol in 100 mL Milli-Q water and 1.5 Eppendorf tubes were autoclaved. 5 mL of 50% glycerol were pipetted onto bacterial cells in the plate and stirred with a sterile loop to ensure the mixture is homogeneous. Then, the mixture was transferred into 2 mL Eppendorf tubes in triplicates. Finally, the tubes were labeled and kept directly at -80°C.

2.7. Molecular characterization of bacterial isolates

2.7.1. Genomic DNA extraction for gram positive bacteria

DNA was extracted from freshly overnight bacterial pure cultures prepared as described in 2.4.1.above. 20 mg of cell pellet obtained by centrifuging 2 mL of the overnight culture for 5 min at 10000 rpm was used as the initial material. The extraction was performed using the DNeasy Plant Mini kit (QIAGEN, Düren, Germany) by following the manufacturer's instructions with some modifications. For Gram positive bacteria with a thick cell wall and high resistance to physical disruption, cells were mixed with 500 µL of buffer AP1 and 5 µL RNase, instead of 400 µL and 4 µL respectively, in Peqlab-tube (Precellys Glas/Keramik-Kit S38), VWR, Darmstadt, Germany. Then, cells in the tubes were homogenized in Precellys-pressure-cycler, as follows: 3 × 40 s at 65,000 rpm 30 s pause. Afterwards, they were incubated on a thermocycler for 10 min at 600 rpm and 95°C instead of 65°C. After cooling them down on the ice for 5 min, they were again homogenized in Precellys-pressure cycler using the same program. Later, the tubes received 160 µL of buffer P3 and incubated for 15 min instead of 130 µL of buffer P3 and 5 min of incubation. Post this treatment, the next steps were done following the protocol that was incorporated in the kit. The extracted genomic DNA was quantified photometrically by using NanoDropR (ND-100 spectrophotometer, Peqlab, Darmstadt, Germany).

2.7.2. Polymerase chain reactions (PCR)

The amplification of the regions harboring the gene of interest, mainly the 16S rRNA gene (1450 bp) for bacterial identification, the *nif*H gene (350bp) to determine potential N_2-fixing bacteria, and the *pqqC* gene (140pb) for phosphate solubilizing activity, was performed in 25 µL reactions containing 12.5 µL Top Taq Mastermix, 5.5 µL PCR water, 2.5 µL of (3.1 pmol μL^{-1}) each primer and 2 µL of template DNA (15 ng μL^{-1}), using a thermocycler (Peqstar of Peqlab, Darmstadt, Germany). The Table 3 describes all the primers used in the present study. In all cases, PCR with water was used as a no template control, and the diazotrophic bacterial strain DSM16656 *Kosakonia radicincitans* (Berger et al., 2015; Ruppel and Merbach, 1995) served as a positive control for the *nif*H gene and negative control for the *pqqC* gene.

For PCR-pqqC temperature profile was as follows: One cycle at 94 °C for 3 min, 96 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s, 2 cycles at 96 °C for 20 s, 62 °C for 30 s, 72 °C for 35 s, 3 cycles at 96 °C for 20 s, 59 °C for 30 s, 72 °C for 40 s, 4 cycles at 96 °C for 20 s, 56 °C for 30 s, 72 °C for 45 s, 5 cycles at 96 °C for 20 s, 53 °C for 30 s, 72 °C for 50 s, followed by 25 cycles at 94 °C for 20 s, 50 °C for 30 s, 72 °C for 1 min and a final elongation step of 72 °C for 5 min.

Table 3: The different primers used in the present study.

Primer names	Forward sequence	Reverse sequence	Sources
16S rRNA gene	5′-GAGTTTGATYHTGGCTCAG-3′	5′-ACGGHTACCTTGTTACGACTT-3′	Mühling et al., 2008
***nif*H gene**	5′-GCIWTYTAYGGIAARGGIGG-3′	5′-AAICCRCCRCAIACIACRTC-3′	Jureava et al., 2006
***pqqC* gene**	5′-GYGTSCGBTTYGCVGTBGA-3′	5′-TARTGYTGSGG CCARCTGT-3′	Anzuay et al., 2013

2.7.3. Gel electrophoresis and PCR product purification

The visualization of PCR-amplified products on agarose gels was done by loading 5 µL (3 ng) of the products on a 1.5% agarose gel containing gel red nucleic acid stain (0.025 µL mL^{-1}) soaked in 1X TAE buffer. All electrophoresis gels were run at 100 A for 30 minutes. A FastRuler 1kb DNA ladder (Thermo Fischer Scientific, Darmstadt, Germany) was used for all agarose gels as a DNA size marker. Purification of PCR products, particularly prior to sequencing, was done using a PCR purification kit (QIAGEN, Düren, Germany) following the standard protocol recommended by the manufacturer.

2.8. Colorimetric method to determine P from filtrated cultures for P solubilization in liquid culture

Available P was determined following the colorimetric molybdate blue method (Bae et al., 1999). Accordingly, 100 µL of supernatant were mixed with 100 µL of 5.5% trichloro acetic acid and incubated for 10 min in a microplate reader (Infinite 200 Pro, Tecan, Männedorf, Switzerland). Afterwards, 100 µL of color reagent were prepared by mixing four volumes of 1.5% (w/v) ammonium molybdate in a 5.5% (v/v) sulfuric acid solution and one volume of a 2.7% ferrous sulfate solution, and added to the sample solution. The intensity of blue color, measured at 700 nm, was directly proportional to the concentration of soluble phosphate in the sample. 5.5% trichloroacetic acid (TCA) was prepared by dissolving 5.5 g trichloroacetic acid in 100 mL distilled water; 1.5% (w/v) ammonium molybdate was prepared by dissolving 1.5 g ammonium molybdate in 100 mL; the preparation of 5.5% (v/v) sulfuric acid was done by dissolving 5.5 mL sulfuric acid in 94.5 mL distilled water, while that the solution of 2.7% ferrous sulfate was obtained by dissolving 2.7 g ferrous sulfate in 100 mL distilled water. A standard calibration curve was generated using a KH_2PO_4 solution (100 mg P L^{-1}) that was prepared by dissolving 0.11 g of KH_2PO_4 in 250 mL distilled water. The pH of the filtrate was determined in each case by using a pH 110 meter (VWR, Darmstadt, Germany).

2.9. Preparation of CAS plate for siderophore production test

The Schwyn and Neilands (1987) method is used to identify isolates able to produce siderophores through the utilization of chrome azurol S (CAS), a blue dye. The ternary complex chrome azurol S/iron (III)/hexadecyltrimethylammonium bromide serves as an indicator. When a strong chelator (as a siderophore) removes the iron from the dye, its color turns from blue to orange. Orange halos around the colonies on blue agar are indicative of siderophores excretion by the bacterial isolate.

To prepare 1 L of CAS blue agar, 4 types of solution were needed (Schwyn and Neilands 1987). CAS assay solutions 1, 2, 3, and 4 were prepared separately. To prepare solution 1, i.e., the Fe-CAS indicator solution, 60.5 mg CAS (Sigma Aldrich, St. Louis, USA) were dissolved in 50 mL water and mixed with 10 mL iron (III) solution (1 mM $FeCl_3 6H_2O$, 10 mM HCl). Under stirring condition, this solution was slowly added to 72.9 mg HDTMA dissolved in 40 mL water. The resultant dark blue liquid was autoclaved and cooled to 50°C. To prepare buffer solution 2, 30.24 g PIPES were dissolved in 750 mL of salt solution containing 0.3 g KH_2PO_4, 0.5 g NaCl, and 1.0 g NH_4Cl and a 50% (w/w) KOH solution was added to raise the pH to the value of the pKa of PIPES (6.8). Solution 2 was autoclaved after adding 15 g of agar, and then cooled to 50°C. For preparing solution 3, 100 mL of KB medium (proteose peptone no. 3.2%, K_2HPO_4 0.115%, $MgSO_4 \cdot 7H_2O$ 0.15%, and glycerol 1.5%) were mixed with 70 mL of a solution containing 2 g glucose, 2 g mannitol, 439 mg $MgSO_4 7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4 H_2O$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4 5H_2O$, 1.2 mg $ZnSO_4 7H_2O$, and 1.0 mg $Na_2MoO_4 2H_2O$. Solution 3 was autoclaved and cooled to 50°C. Solution 4 was a 10% (w/v) casamino acid solution, the nitrogen source. 30 mL of 10% (w/v) casamino acid solution was filter-sterilized. All the four solutions were mixed and the resulting mixture was poured into petri dishes. After solidifying, bacterial suspensions prepared in standard nutrient broth were adjusted to OD 0.2 at 620 nm, then 10 µL were dropped onto the center of the CAS-blue agar plate and incubated at 28°C for 72h. Color change reaction on the CAS-blue agar plate to orange, indicated the presence of siderophore after chelation of the bound iron. The experiment was performed once with three replicates for each bacterial strain.

2.10. Seed germination

2.10.1. Seed sterilization

All seeds used for the *in vitro* seed germination assay or *in vivo* experiments in greenhouse were surface sterilized following a well-established protocol (Johnston-Monje and Raizada, 2011), but with some modifications. The Triton X-10 detergent step was not included. To soften seeds, they were soaked in distilled water for 48 h. After removal of the water by draining, the soften seeds were washed two times with 3% sodium hypochlorite (NaOCl) for 5 min, then rinsed three times with autoclaved, distilled water, before being washed in 95%

ethanol for 10 min. The ethanol wash was drained, and seeds rinsed six times with autoclaved, distilled water. To check for surface sterility, few seeds were momentarily placed on sterile water agar plates and these plates were incubated for 5 days at 28°C.

2.10.2. Seed inoculation

Bacterial inoculum for each selected strain used for the *in vivo* experiments was prepared as described above. The cultures were suspended in 0.05 M NaCl buffer solution and the inoculum concentration was always adjusted to 10^8 cells mL^{-1} to prevent seeds negative response to higher bacterial cell density. Surface-sterilized seeds were inoculated by immersion in 10 mL of the respective bacterial suspension (microbial treatments) for 15 min. Seeds for the control treatments were treated in the same way but with a bacteria-free 0.05 M sterilized NaCl buffer solution. Inoculated seeds were sown immediately after the inoculation procedure.

2.10.3. Sowing

Seeds were sown and germinated in quartz sand in green plastic trays. Two trays were used for each treatment. First, a porous tray was covered with a layer of laboratory paper towels to prevent sand leakage the porous trays bottoms and placed into a non-porous tray as a collector for the water that drains from the top tray. Each tray was then filled with quartz sand, leveled and lightly watered with osmose water. The treated seeds were placed in shallow wells on the surface of the wet sand in their respective treatment trays. The seeds were covered with a thin layer of sand achieved by straining quartz sand over the trays through a fine sieve and then covered with a transparent cap to retain moisture loss. During the germination process, the trays were watered once every day with osmose water. The trays were placed in the phyto-chamber for two weeks for seedling germinations. The phyto-chamber was set up with the following germination program: first 72 hours without light and followed by a light provision 12 hours a day for eleven days. The temperature in the phyto-chamber was set at 25°C during the day and 20°C during the night.

2.11. Plant greenhouse experiments

2.11.1. Transplanting and seedling inoculation

After the germination period, on the 14th day, the plants were transplanted to achieve one seedling per pot. Seedlings were potted in pots containing 1 L of mixed quartz sand and vermiculite (1/1). In all inoculated pots, seedlings were transferred to a pit and finely covered with soil and re-inoculated the following day with 2.5 mL of the respective bacterial suspension or with 0.05 M sterilized NaCl solution for the control treatments (negative and positive). After transplanting, pots were completely randomized and kept on greenhouse benches for the entire growing season.

2.11.2. Greenhouse growth conditions and postharvest

For *in vivo* experiments conducted using tomato and maize as model plants, plant growth was documented over six weeks after transplanting in a greenhouse at a day/night temperature 25/23°C and 75% air humidity throughout the day. For experiments conducted during winter season, additional light was provided to plants. The pots were completely randomized and re-positioned weekly to minimize the environmental effects. At the harvest, plants were removed from the pots and roots were thoroughly washed in tap water and deionized water to remove attached soil. Shoot height and root length (root depth) were measured by using a ruler. Then, root and shoot were separated. Shoot, root and total fresh biomass and shoot height were determined immediately. Then, samples were oven-dried at 60°C for 72h and dry weights were recorded.

2.11.3. Modified Hoagland nutrient solution

Several stock solutions were prepared for the Hoagland solution (Hoagland and Arnon 1950): 0.2 M NH_4NO_3 made by dissolving 16 g in 1 L distilled water, 0.1 M $Ca(NO_3)_2x4H_2O$ was prepared by dissolving 24 g in 1 L distilled water, 1 M $CaCl_2x6H_2O$ was made by dissolving 219 in 1 L distilled water, 0.5 M K_2SO_4 by dissolving 87 g in 1 L distilled water, 0.05 M KNO_3 by dissolving 6 g in 1 L distilled water, 1 M $MgSO_4x7H_2O$ made by dissolving 246 g in 1 L distilled water, 1M KH_2PO_4 made by dissolving 136 g in 1 L distilled water, 0.02 M $FeCl_2x6H_2O$ made by dissolving 5 g in 1 L distilled water, and a micronutrient solution made by dissolving 2.86 g H_3BO_4, 1.81 g $MnCl_2x4H_2O$, 0.22 g $ZnSO_4x7H_2O$, 0.06 g $CuSO_4.5H_2O$ and 0.01 g $(NH_4)_6Mo7O_{24}x4H_2O$ dissolved in 1 L distilled water. To make the half-strength modified Hoagland nutrient solution, 59 ml from the 0.2 M NH_4NO_3 stock, 4 mL from the 0.1 M $Ca(NO_3)_2x4H_2O$ stock, 5 mL from the 0.5 M K_2SO_4 stock, 6 ml from the 0.05 M KNO_3 stock, 2 mL from the 1 M $MgSO_4x7H_2O$ stock, 1 mL from the 1 M KH_2PO_4 stock,1 mL from the Fe-EDTA stock, and 1 mL from the micronutrient stock were added into a laboratory bottle, and the volume was adjusted to 1 L with distilled water.

2.12. Plant nutrient analysis

P, K and Na in plant samples were determined by means of inductively coupled plasma optical emission spectrometry (ICP-OES) after microwave-heated pressure digestion. The principle of this is based on the separation of quantities and trace elements released from the accompanying matrix. Oven-dried tissues were finely ground using a Fritsch Pulverisette plant grinder (Fritsch Pulverisette, Oberstein, Germany). The ground and homogenized material was digested according to the standard protocol (Kirkpatrick and Bishop, 1971). Briefly, 0.5 g of the tissues was mixed in a TFM or PFA tube with 65% nitric acid and 30% hydrogen peroxide. These preparations were placed in a microwave-heated pressure digestion system, MARS 5 Xpress (CEM GmbH, Kamp-Lintfort, Germany), and the elements were extracted from the matrix

using a defined temperature program. Phosphorus was measured at a wavelength of 178.284 nm and axial observation, potassium was measured at a wavelength of 766.490 nm and radial observation, and sodium at a wavelength of 589.592 nm and radial observation, by using iCAP 7400 (Thermo Fisher Scientific GmbH, Dreieich, Germany).

2.13. Statistical analyses

All data sets were tested for parametric test assumption for normal distribution using the Shapiro-Wilk test and for homogeneity of variances using the Levene test. All data sets that fulfilled the parametric test assumptions were analyzed statistically using the analysis of variance test (ANOVA). In all cases, mean comparison between treatments was conducted using the Tukey HSD. Significance was determined at 5% ($p \leq 0.05$) probability level, and significantly different means were indicated by different letters. All the statistical calculations were performed using SigmaPlot software version 12.3 (Systat Software GmbH, Erkrath, Germany).

CHAPTER 3: ISOLATION AND CHARACTERIZATION OF PLANT GROWTH-PROMOTING RHIZOBACTERIA ASSOCIATED WITH MAIZE GROWN IN CAMEROON.

This chapter is based on the following published articles:

G. V. Tchuisseu Tchakounté, B. Berger, S. Patz, H. Fankem, S. Ruppel (2018). Community structure and plant growth-promoting potential of cultivable bacteria isolated from Cameroon soil. *Microbiological Research*, 214, 47-59

G. V. Tchuisseu Tchakounté, B. Berger, S. Patz, H. Fankem, S. Ruppel (2018). Data on molecular identification, phylogeny and in vitro characterization of bacteria isolated from maize rhizosphere in Cameroon**.** *Data in Brief*, 19, 1410-1417

3.1. Abstract

Exploiting native plant growth-promoting rhizobacteria (PGPR) in Cameroonian agro-ecosystems provides a means to improve plant–microbe interactions that may enhance ecosystem sustainability and agricultural productivity in an environmentally eco-friendly way. Consequently, we aimed to investigate the community structure and functional PGPR diversity of maize grown in Cameroon. Native bacteria isolated from Cameroon maize rhizosphere soil were identified by partial 16S rRNA gene sequencing and screened for traits particularly relevant for Cameroon low-fertility soil conditions, such as their abilities to tolerate high concentrations of salt, and their plant growth- promoting potential. Genetic and functional diversity was characterized according to their phylogenetic affiliation. A total of 143 bacteria were identified and assigned to 3 phyla (*Actinobacteria*, *Firmicutes* and *Proteobacteria*), 13 families and 20 genera. *Bacillus* (31.5%), *Arthrobacter* (17.5%), and *Sinomonas* (13.3%) were the most abundant genera identified among all the isolates. Based on their *in vitro* characterization, 88.1% were salt tolerant at 2% NaCl, but only 16.8% could tolerate 8% NaCl, 50.4% solubilized phosphate, 10.5% possessed the *nifH* gene, and 19.6% produced siderophores. Six isolates affiliated to the most abundant genera identified in this work, *Bacillus* and *Arthrobacter,* carrying multiple or only single tested traits were selected to evaluate their growth- promoting potential in an *in vitro* maize germination assay. Three strains possessing multiple traits induced significantly increased hypocotyl and root length, and improved the germination rate of maize seeds compared to non-inoculated control seeds. Our results indicate the potential of selected indigenous Cameroon rhizobacteria to enhance maize growth.

3.2. Introduction

Maize is a strategic crop in terms of food security and economic profitability. The plant is mainly grown by small-scale subsistence farmers (Epule and Bryant, 2014). Although maize is cultivated in all five agro-ecological zones of Cameroon and is the most affordable crop in terms of market price and cost of seeds, grain yields often remain low compared with local food demand. One reason is that Cameroonian soils are generally low in fertility, particularly lacking phosphorus (P) and nitrogen (N) (Fankem et al., 2006). Low soil fertility and other issues such as salinity, soil acidity and water stress are the major limiting factors in Cameroonian agriculture, since nutrient deficient and salt-stressed soils are known to severely suppress plant growth and crop productivity. Considering the low corn yields still pervasive in farmers' fields, increasing maize production is an urgent task to secure food supplies in the country.

In particular, the P fixation capacity of soils is a critical problem that leads to low soil fertility. P is a component of key molecules such as nucleic acids, phospholipids, and ATP. It is also involved in controlling key enzyme reactions and regulating metabolic pathways, and consequently, plants cannot grow without a reliable supply of this nutrient (Theodorou and Plaxton, 1993). Like many tropical and subtropical soils, Cameroonian soils are predominantly acidic; a high content of iron and aluminum ions effectively react with P in such soils. Consequently, about 75% of P applied as chemical fertilizer or natural rock phosphate is converted into insoluble complexes (Gyaneshwar et al., 2002), making the P-deficiency in soil difficult to overcome. Seeking a solution, soil microorganisms could contribute efficiently to improving soil fertility. Besides the other plant growth-promoting (PGP) traits, the ability to solubilize different kinds of synthetic inorganic phosphate and natural rock phosphates is particularly crucial in the selection of suitable bacterial candidates for Cameroonian agriculture. Moreover, seeking PGPR possessing novel traits such as salt tolerance will improve salinity management in these nutrient deficient and salt affected soils.

Despite the potential benefits of using PGPR to enhance crop productivity and improve crop protection under normal and salt stressed conditions (Ahemad and Kibret, 2014; Sharma et al., 2016), these strategies are still largely untapped in the effort to improve maize production in Africa. Especially in Cameroon, little information is available on the occurrence and use of PGP bacteria, and no research has been devoted so far to studying indigenous PGPR associated with maize grown in the country. It is important to study native microbial communities associated with plants in order to understand their ecological role in specific environments (Cavaglieri et al., 2009). Studies have shown that to maximally exploit the plant-bacteria association effective bacteria must be selected in plant studies that take specific ecological conditions into consideration, e.g. crop management, soil type and climate (Perez-Montano et al., 2014). Under such conditions, knowledge about the native bacterial populations, their

identification and their implications for plant physiology, is required for improving management practices regarding plant nutrition and defense. The use of indigenous PGPR for creating bacterial inoculants can be an advantage since these organisms easily acclimatize to the respective environmental conditions and may more easily establish the plant-microbe interaction (Verma et al., 2013).

In this study, we aim to isolate and characterize rhizobacteria from maize cultivated in the region of Cameroon with the highest maize cropping density. We hypothesized that the rhizosphere of maize grown in Cameroon harbors a high diversity of cultivable bacteria exhibiting multiple plant growth– promoting and salinity tolerance activities. The main goal of our study was to: (i) isolate a wide range of native cultivable bacterial strains from the maize rhizosphere in Cameroon; characterize the isolates based on their attributes and phylogenic affiliation (partial 16S rRNA gene sequence); (iii) evaluate their *in vitro* potential for salinity tolerance, synthetic and natural inorganic phosphate solubilization, atmospheric nitrogen (N_2) fixation by searching for the presence of *nifH* gene and siderophores production, and (iv) assess the bacterial *in vitro* effect on maize seedlings at the germination stage.

3.3. Materials and methods

3.3.1. Study site and sample collection

Soil samples with the characteristics described in Table 4 were collected from maize rhizospheres at a farm in the Ngaoundal locality (6° 30" North, 13° 16" East) in the high Guinea savanna zone II, where the southern plateau raises northward to the grassy, rugged Adamawa Plateau (Fig. 6). This feature stretches from the western mountain area and forms a barrier between the country's north and south. Its average elevation is 1,100 meters, and temperatures range from 22°C to 25°C with high rainfall. The spot was chosen for sample collection because it is the most cultivated maize region in the country. Rhizosphere soils adhering to maize roots at a depth of 10 to 20 cm were collected from 20 randomized plant rhizospheres of the farm. The samples were mixed to form a composite sample, then packed in a sterile plastic bag and immediately taken to the laboratory. The soil was passed through a 4 mm sieve to eliminate coarse rock and plant material, thoroughly mixed to ensure uniformity, and stored at 4°C prior to use. A subsample about 0.5 kg was air dried and passed through a 2 mm sieve for chemical analysis as described in chapter 2.

Table 4: Elemental composition of the soil sample (mean values of four replicates)

	mg kg^{-1}					
pH	P_{dl}	K_{dl}	Mg	N_t	C_t	**C/N ratio**
5.6	49	62	81	2380	27930	11.7

dl = double lactate extractable

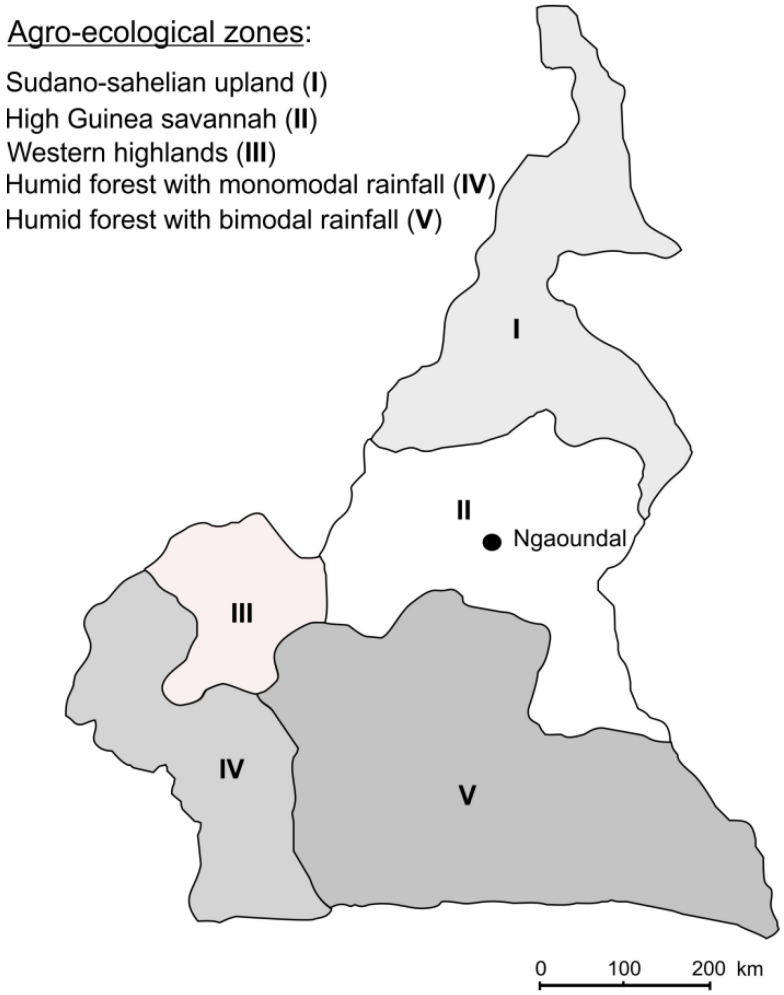


Figure 6. Location of the sampling site: different agro-ecological zones of Cameroon and the sampling site (Ngaoundal) represented as a black dot in zone II.

3.3.2. Isolation, purification and conservation of bacterial isolates

The isolation of microorganisms was assessed in non-selective nutrient agar (NA) medium (Standard nutrient agar I, Carl Roth, Germany) containing 6 g NaCl, 3 g yeast extract, 15 g peptone, 1 g glucose, 12 g agar-agar L^{-1}, pH 7. Four independent replicates were analyzed as

follows: Ten g soil was homogenized in Erlenmeyer flasks in 90 mL of sterile buffer (NaCl, 0.05 M) by shaking at 290 rpm for one hour. This solution was ten-fold diluted (10^{-1} to 10^{-7}) and 0.1 mL aliquots of dilutions 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were plated on nutrient agar media in triplicates. The plates were incubated at 28°C for five days. Individual colonies were morphological characterized based on their size, shape, color, surface, margin and elevation (Santos et al., 2015), grouped and quantified. Twenty colonies of each morphotype were picked and streaked on the NA media for further purification. Pure strains were stored in 50% sterile glycerol at -80°C.

3.3.3. Molecular identification and phylogenetic analysis of bacterial isolates

The extraction of genomic DNA from overnight pure bacterial culture grown in nutrient broth (Standard nutrient broth I, Carl Roth, Germany) at 28°C was performed following the above described protocol. The DNA concentration was determined photometrically at 260 nm and quality was checked by the A_{260}/A_{280} ratio calculation being above 1.9 and the A_{320} measurement nearly 0 using NanoDropR (ND-100 spectrophotometer, Peqlab, Germany). The genomic DNA extracted from all isolates was used for partial 16S rRNA gene amplification using 16S rDNA universal primers: 9bfm (5′ -GAGTTTGATYHTGGCTCAG-3′) and 1512R (5′-ACGGHTACCTTGTTACGACTT-3′) (Muhling et al., 2008). Amplification reactions were performed in 25 µL reactions containing 12.5 µL Top Taq Mastermix, 5.5 µL PCR water, 2.5 µL of (3.1 pmol μL^{-1}) each primer and 2 µL of template DNA (15 ng μL^{-1}). Amplification of 16S rRNA gene portions was carried out in a thermocycler (Peqstar of Peqlab, Germany). The conditions were as follows: an initial activation step at 96°C for 4 min, followed by 30 cycles comprising denaturation at 96°C for 1 min, primer annealing at 56°C for 1 min, primer extension at 74°C for 90 s and finally extension at 74°C for 10 min.

The purified PCR products were sequenced using the DNA sequencing service of Eurofins Genomics, Germany. The bacterial 16S rDNA nucleotide sequences (mean length of 790 bp) were aligned with known sequences in the National Center for Biotechnology Information (NCBI http://blast.ncbi.nlm.nih.gov) and Ribosomal Database Project (RDP) databases using BLASTn. Sequences of all related species were retrieved to derive the nomenclature of the isolates. Multiple sequence alignments with the most closely related bacterial sequences were performed using Muscle (https://www.ebi.ac.uk/Tools/msa/muscle/) and phylogeny was inferred by the Maximum Likelihood approach based on the Tamura 3-parameter model and the neighbor-joining method (Saitou and Nei, 1987), using Mega 7 version 7.0.21 (http://www.megasoftware.net/). Phylogenetic tree topology based on re-sampling 1000 times the neighbor joining data set was evaluated by bootstrap analysis. To better understand and facilitate the visualization of functional diversity between and within genera, Interactive Tree

Of Life web-based tool (http://itol.embl.de) was used to display the different traits harbored by each bacterial isolate on the phylogenetic trees (Letunic and Bork, 2016).

3.3.4. In vitro characterization of bacterial isolates

Bacterial tolerance to salinity

The intrinsic tolerance of bacterial isolates to salinity was evaluated by observing the bacterial growth on Standard I Nutrient agar (Carl Roth, Germany) amended with various concentrations of NaCl (2, 4, 6, and 8% w/v). Control plates were also maintained with 0.05% NaCl (w/v). The isolates were streaked on plates and placed in an incubator at 28°C. The plates in triplicate for each strain were examined for bacterial growth after 72 h, and the supplemented plates were compared with controls without salt addition (Upadhyay et al., 2009).

Phospate solubilization assay

The ability of isolates to solubilize seven different inorganic phosphate sources (tricalcium phosphate, hydroxyapatite, Malian rock phosphate (RP), Cameroonian RP, Algerian RP, Mexican RP, Moroccan RP) was assessed on plates filled with the National Botanical Research Institute's Phosphate growth medium (NBRIP) (Nautiyal, 1999). The NBRIP was modified as follows, containing per liter of distilled water: 20 g glucose, 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ and one phosphate type at 5 g, agar-agar 15g, plus 0.5% bromocresol green, pH 7.5 (Fankem et al., 2014). A stock solution of 0.5% dye was prepared by dissolving a corresponding weight of bromocresol green into 70% ethanol and the final pH adjusted to 6.5 with 1M KOH. 10 µl of each bacterial suspension of adjusted OD to 0.2 at 620 nm were then transferred onto a single point of compartmented Petri dishes. Plates in triplicate for each bacterial isolate were incubated at 28°C for 7 days and positive isolates recorded through the yellow halo zone surrounding the bacterial colony.

Amplification of the nifH gene

Potential N_2-fixing bacteria were determined by searching for the presence of the *nif*H gene, the marker gene for biological nitrogen fixing ability (Juraeva et al., 2006). Universal primers 19F (5′-GCIWTYTAYGGIAARGGIGG-3′) and 366R (5′-AAICCRCCRCAIACIACRTC-3′) (Juraeva et al., 2006), 50 ng of template DNA, 5.5 µL PCR water, 2.5 µL of (3.1 pmol μL^{-1}) each primer and 12.5 µL of QIAGEN Top Taq Mastermix were used to amplify nifH PCR fragments (350 bp). The following PCR reaction conditions were applied: initial activation step at 94°C for 4 min, followed by 35 cycles comprising denaturation at 94°C for 30 s, primer annealing at 55°C for 1 min, primer extension at 72°C for 75 s and finally extension at 72°C for 10 min in a thermocycler (Peqstar of Peqlab, Germany). PCR with water was used as a no template control, and the diazotrophic bacterial strain DSM16656 Kosakonia radicincitans (Berger et al., 2015; Ruppel and Merbach, 1995) served as a positive control.

Siderophores production assay

Siderophores production by bacterial isolates was determined following the universal assay of Schwyn and Neilands using CAS-blue plates as described by Ji et al. (2014). First bacterial suspensions were adjusted to OD 0.2 at 620 nm, then 10 µL were dropped onto the plate and incubated at 28°C for 72 h. Color change reaction on the CAS-blue agar plate to orange, indicated the presence of siderophores after chelation of the bound iron. The experiment was performed once with three replicates for each bacterial strain.

3.3.5. Seed germination bioassay

Six selected bacterial strains (V1, V39, V54, V62, V64, V84) presenting different abilities for the specific traits tested and belonging to *Arthrobacter* and *Bacillus* genera were assessed for their ability to promote maize growth at the germination stage. Maize seeds variety "LUIGI CS" (Caussade, France) were surface sterilized as described earlier by Johnston-Monje and Raizada (2011). Bacterial inoculums were prepared by transferring a single pure colony of each bacterial isolate into 100 mL Erlenmeyer flasks containing 50 mL nutrient broth and grown in flasks on a rotary shaker (180 rpm) at 28°C for 24 h. Bacterial cells were harvested and washed three times in 0.05 M sterilized NaCl solution after centrifugation (10,000 x *g*) for 15 min at 4°C and finally suspended in 0.05 M sterilized NaCl solution until the population reached 10^8 colony forming units (CFU) mL^{-1}. Six surface sterilized seeds were dipped in bacterial cultures (microbial treatments) or 0.05 M NaCl (control treatments) for 15 min and then dried in a laminar flow bench for 1 to 2 h at room temperature. Seeds were placed in petri dishes lined with sterilized moistened filter paper. The plates were incubated for 5 days at 28°C. Germinated seeds were counted at day 5. The germination rate, hypocotyl and root lengths were recorded and the vigor index (VI) was calculated using the following formula: VI = (mean hypocotyl length + mean root length) x % germination. This experiment was carried out three times.

3.2.6. Statistical analyses

Statistical analyses were performed using Sigma plot software version 12.3. Data were subjected to the one-way analysis of variance to find differences between treatments and the control. Mean comparison between treatments was conducted using the Tukey HSD test. Significance was determined at 5% ($p \leq 0.05$) probability level, and significantly different means were indicated by different letters.

3.4. Results

3.4.1. Cultivable bacteria inhabiting maize rhizosphere in Cameroon: Actinobacteria, Firmicutes and Proteobacteria

We were able to recover maize rhizosphere soil bacterial isolates on NA medium and grouped them into 31 morphotypes according to their colony characteristics. Four morphotypes represented approximately 10^4, while some could reach values of approximately 10^7 CFU per gram of soil, which comprises a total population captured of 6.64 x 10^7 CFU per gram of soil. We randomly selected and purified a total of 156 isolates from all the 31 different morphotypes, and designated them by letters and progressive numbers of isolation (Table 15 see appendix).

Based on their partial 16S rRNA gene sequencing, 143 isolates provided good-quality sequences (mean length of 790 bp) and were affiliated to bacterial species using phylogenetic classification based on NCBI and RDP databases. We further investigated the phylogenetic assignment of all isolates by constructing a phylogenetic tree using representative sequences obtained from NCBI and RDP databases (Fig. 7 and Fig.33 in appendix). We classified all the strains into three Phyla: *Actinobacteria*, *Firmicutes* and *Protebacteria*. We could set up 17 clades from the 143 isolates based on their 20 different genera with high bootstrap values (Fig.7; Fig 8).

Representative strains of ***Actinobacteria*** were placed in 6 major clades (marked in brownish to yellowish colors in Fig.6). Except for isolates belonging to *Amycolatopsis*, *Mycobacterium*, *Kitasatospora* and *Streptomyces* genera, and to *Leifsonia* and *Microbacterium* genera, which clustered together each forming one clade, all other clades comprised isolates belonging to the same genus. Isolates V76, VA39, VA125, V98 and V9 were respectively affiliated to *Micrococcus yunnanensis*, *Micrococcus aloeverae*, *Sinomanas atrocyaneus*, *Microbacterium azadiractae* and *Leifsonia shinshuensis*.

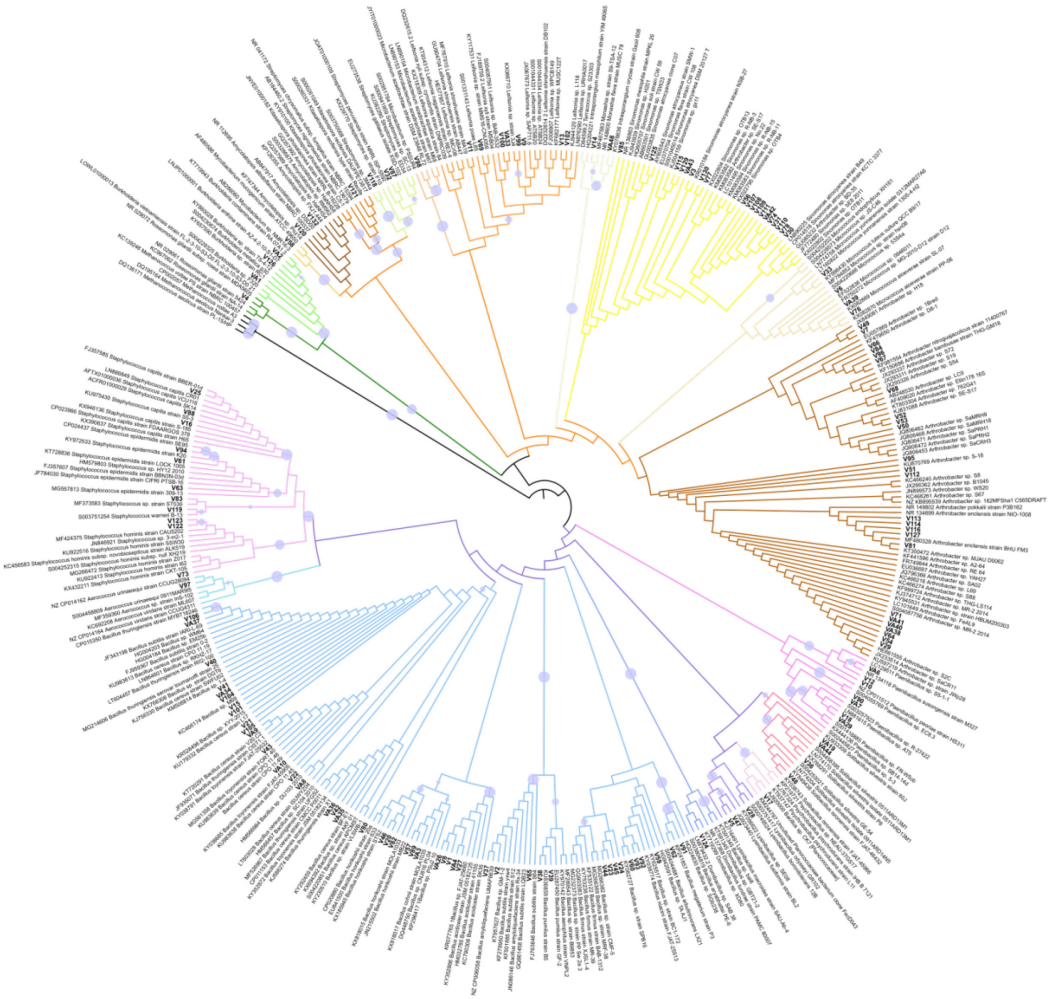


Figure 7. Bootstrap consensus tree based on 16S rDNA sequences revealing phylogenetic classification of the 143 isolates: The colors indicate different bacterial genera. The isolates between sequences are represented in bold. The Maximum Likelihood tree was constructed using the Tamura 3-parameter model and the neighbor joining method, with the bootstrap analyses of 1000 replicates. Only bootstrap values equal and greater than 90% are displayed as circles with increasing size up to 100%. *Methanococcus* ssp. was used as outgroup.

The ***Firmicutes*** phylum comprised 9 clades (marked in purplish to pinkish colors in Fig. 7). Each clade constituted isolates pertaining to the same genus, with the exception of *Bacillus*, *Solibacillus*, unclassified *Planococcaceae* and *Lysinibacillus*. Isolates belonging to the *Bacillus* genus were split into three clades and were affiliated to six different species. Isolates of the three other genera formed one group, even though they do not all belong to the same family. Two isolates, V88 and V119, were almost identical to the type of strains *Staphylococcus capitis* and *Staphylococcus warneri*. V1 was closely related to *Bacillus acidiceler*, VA8 and V21 to *Bacillus cereus*, V39 and V38 to *Bacillus pumilus*, and V62 to *Bacillus megaterium*, while one isolate V28 clustered with *Lysinibacillus odysseyi*. Isolate V17 clustered to uncultured *Planococcaceae* bacteria despite showing high similarity to *Bacillus* sp. CK7, classified as *Planococcaceae* in the RDP database.

For ***Proteobacteria*** (greenish colors in Fig. 7), this phylum was divided into two clades comprising isolates belonging to *Roseomonas* and *Burkholderia* genera. Isolate V4 was close to *Roseomonas gilardi*, while VA2 was similar to *Burkholderia contaminans*.

To summarize the above results, sequences of the bacterial isolates were assigned to 20 distinct genera, distributed in 13 families and 3 Phyla: *Actinobacteria*, *Firmicutes* and *Proteobacteria* (Fig. 8). The most abundant bacterial genera found among isolates were *Bacillus* (45) and *Arthrobacter* (25) representing 31.5% and 17.5% of the total isolates, respectively, followed by *Sinomonas* (19), *Staphylococcus* (11), *Leifsonia* (8) and *Paenibacillus* (7) belonging to *Bacillaceae*, *Micrococcaceae*, *Staphylococcaceae*, *Microbacteriaceae* and *Paenibacilaceae* families, respectively.

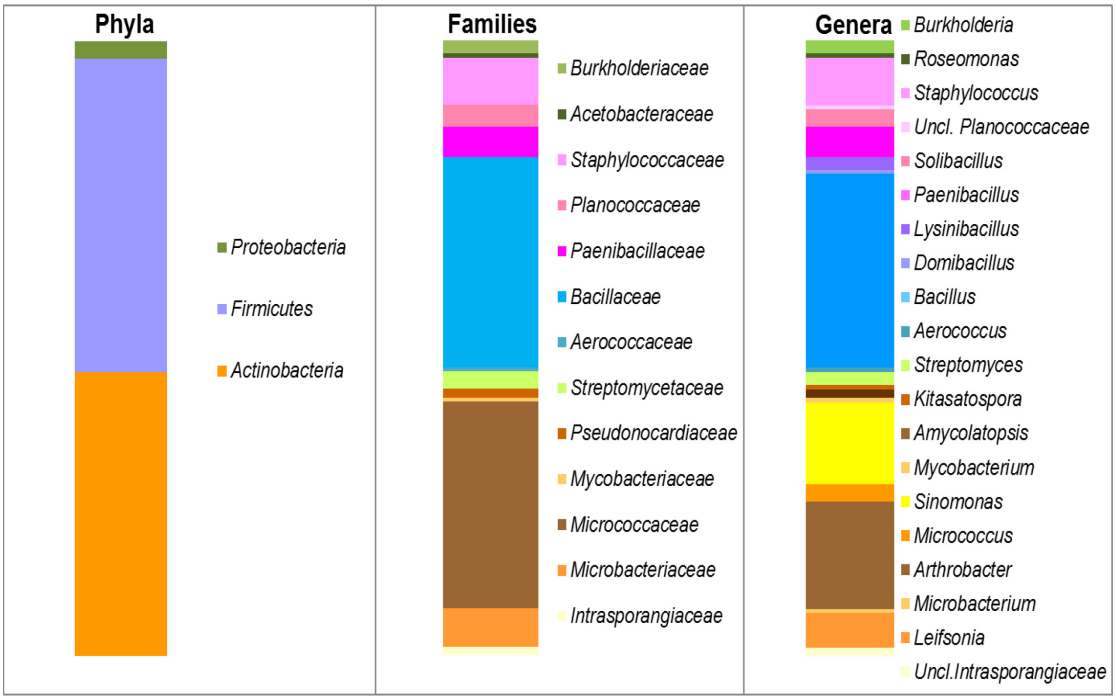


Figure 8. Taxonomic affiliations based on partial 16S rDNA sequencing analysis: the relative abundance of cultivable bacteria inhabiting maize rhizosphere is shown at phylum, family and genus levels. The different color shades represent the dominance of each phylum, each family and each genus at the respective level. Uncl. = unclassified.

Besides these six genera, the rhizosphere bacterial community analyzed also comprised 12 other gram positive bacterial genera, all together accounting for 16.8% of all isolates, with a total number of representative strains ranging from one to four isolates, including various genera such as *Micrococcus* (4), *Lysinibacillus* (3) and *Domibacillus* (1), *Solibacillus* (3) and unclassified *Planococcaceae (1)*, *Streptomyces* (3) and *Kitasatospora* (1), *Amycolatopsis* (2), unclassified *Intrasporangiaceae* (2), *Aerococcus* (1), *Microbacterium* (1), and *Mycobacterium* (1) members of these respective families: *Micrococcaceae*, *Bacillaceae*, *Planococcaceae*, *Streptomycetaceae*, *Pseudonocardiaceae*, *Intrasporangiaceae*, *Aerococcaceae*,

Microbacteriaceae and *Mycobacteriaceae*. Gram negative bacteria were represented by four strains: *Burkholderia* (3) and *Roseomonas* (1) genera (Fig. 8) pertaining to *Burkholderiaceae* and *Acetobacteraceae* families.

3.4.2. Bacterial isolates harbor different functional traits: most common were salinity tolerance and phosphate solubilization

We screened all identified bacterial strains *in vitro* for their ability to tolerate different concentrations of salt (2 - 8% NaCl); to solubilize seven different inorganic phosphate compounds typically used as fertilizers (tricalcium phosphate, hydroxyapatite, and five rock phosphates from different origins: Mali, Cameroon, Algeria Mexico and Morocco); for atmospheric nitrogen fixation by possessing the *nifH gene*, and to produce siderophores. Out of 143 bacterial isolates, 131 (91.6%) displayed at least one of the targeted traits tested, distributed in 19 out of 20 genera, in all families and in all phyla (Fig. 9). The most dominant trait among the isolates was salinity tolerance with an abundancy of 88.1%, followed by phosphate solubilization (50.4%), siderophore production (19.6%), and the presence of the *nifH* gene (10.5%; Table 5).

Table 5: Occurrence and characterization of bacterial isolates for salinity tolerance, phosphate solubilization, *nifH* gene presence and siderophore production at the genus level

Genus	Number total of isolates	Number of isolates with at least one PGP trait	Salt Tolerance (>=2% NaCl)	Total P Solubilization	Presence of n*ifH* gene	Siderophore production
Aerococcus	1	1	1	0	0	0
Amycolatopsis	2	1	1	1	0	0
Arthrobacter	25	25	24	19	7	2
Bacillus	45	39	38	20	3	13
Burkholderia	3	3	2	0	0	1
Domibacillus	1	0	0	0	0	0
Kitasatospora	1	1	0	1	0	1
Leifsonia	8	7	7	3	1	1
Lysinibacillus	3	3	3	0	1	0
Microbacterium	1	1	1	1	0	0
Micrococcus	4	4	4	3	0	1
Mycobacterium	1	1	1	0	0	1
Paenibacillus	7	7	7	7	2	1
Roseomonas	1	1	0	1	0	1
Sinomonas	19	19	19	12	0	0
Solibacillus	4	4	4	0	0	0
Staphylococcus	11	11	11	3	1	5
Streptomyces	3	1	1	1	0	0
Uncl. Intrasporangiaceae	2	1	1	0	0	1
Uncl. Planococcaceae	1	1	1	0	0	0
Total 20	143	131	126	72	15	28
Percentage (%)		(91.6)	(88.1)	(50.4)	(10.5)	(19.6)

Uncl. = unclassified.

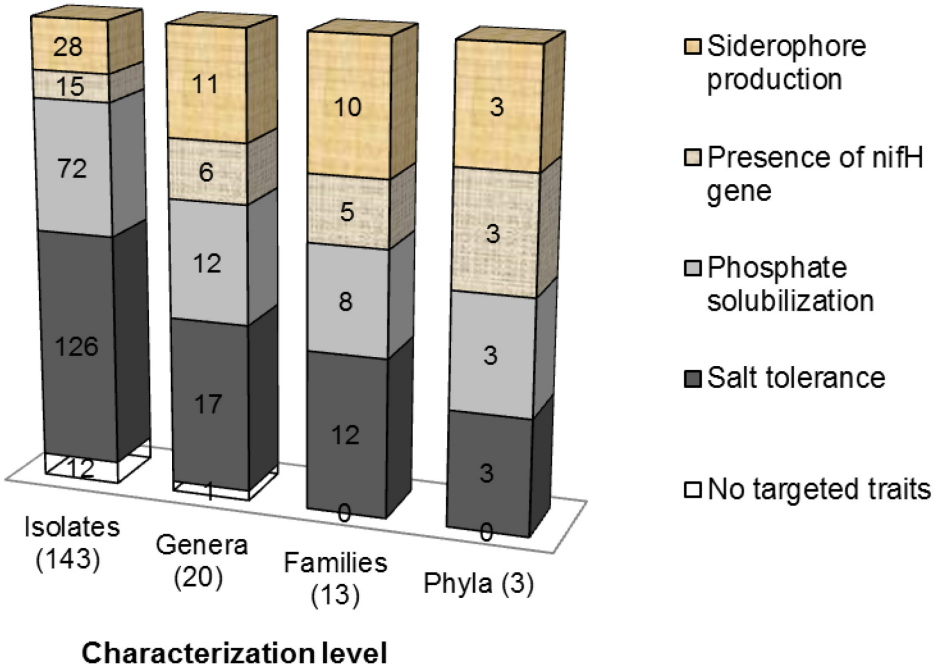


Figure 9. The most common functional traits: distribution of salt tolerance, phosphate solubilization, presence of *nifH* gene for atmospheric nitrogen fixation and siderophore production activity found within all isolates, families, genera and phyla. The number in parenthesis indicates the total number of each taxonomic unit. The number in the different color shades represents the number of each taxonomical unit possessing each trait among the total number.

Salinity tolerance was the most common trait in all the isolates except members of *Domibacillus*, *Kitasatospora* and *Roseomonas* genera; all other genera (17 out of 20) distributed in 12 families had at least one isolate able to tolerate 2% NaCl (Fig. 9). However, the number of salt-tolerant isolates decreased with increasing NaCl concentrations in the medium. In detail, 64.3% (92/143) of the strains were able to tolerate 4% NaCl, 32.9% (47/143) could tolerate 6%, and only 16.8% (24/143) were able to grow on plates supplemented with 8% NaCl, with most isolates, 10 out of 24, belonging to the *Bacillus* genus (Table16, see appendices).

The ability to solubilize phosphate was the second most common feature of the isolates, regardless of the type of phosphate source, and occurred in isolates of 12 genera (*Amycolatopsis*, *Arthrobacte*r, *Bacillus*, *Kitasatospora*, *Leifsonia*, *Microbacterium*, *Micrococcus*, *Paenibacillus*, *Roseomonas*, *Sinomonas*, *Solibacillus*, *Staphylococcus* and *Streptomyces*), grouped into eight families (Fig. 9; Table 17, appendix). Tricalcium phosphate with 46.2% (66/143) was the phosphate source most easily solubilized among the different inorganic phosphate sources tested, followed by Malian RP 43.4% (62/143), Cameroonian RP and hydroxyapatite 29.4% (42/143). In contrast, Moroccan RP with only 2.8% (4/143) was the most recalcitrant phosphate source (Table 17 see appendix). Isolates identified as phosphate

solubilizers mainly belonged to the genera *Bacillus* 27.8% (20/72) and *Arthrobacter* 26.4% (19/72; Table 17, see appendices).

The presence of the *nifH* gene and siderophores production was the rarest traits among the isolates. *nifH* gene could be detected in isolates of six genera: *Arthrobacter*, *Bacillus*, *Leifsonia*, *Lysinibacillus*, *Micrococcus* and *Paenibacillus* pertaining to five families (*Bacillaceae*, *Microbacteriaceae*, *Micrococcaceae*, *Paenibacillaceae* and *Streptomycetaceae*), and the ability to produce siderophores was found in isolates of 10 genera *(Arthrobacter*, *Bacillus, Burkholderia*, *Kitasatospor*a, *Leifsonia, Micrococcus, Mycobacterium*, *Paenibacillus*, *Roseomonas* and *Staphylococcus*) members of ten families. While the *Arthrobacter* genus presented the greatest number of isolates 46.7% (7/15) among potential N_2- fixing bacteria, the *Bacillus* genus had the most isolates with siderophores production capacities 46.4% (13/28; Table 18, see appendices).

We also assessed the variability of the bacterial isolates in displaying the different traits tested by focusing only on the six most abundant genera found among the isolates. The different bacterial genera showed different efficiencies in strains expressing single traits (Fig. 10). For example, the percentage of salinity tolerance at 2% NaCl varied from 84.4% in *Bacillus* to 100% in *Paenibacillus*, *Sinomonas* and *Staphylococcus* strains. Concerning the PGP traits, strains showing phosphate solubilization ranged from 27.3% in *Staphylococcus* to 100% in *Paenibacillus*, while *nifH* gene was detected in 6.7% of *Bacillus*, 28% of *Arthrobacter* and 28.6% of *Paenibacillus* strains. Finally, siderophores were produced by 45.5% of *Staphylococcus*, 28.8% of *Bacillus* and only 8% of *Arthrobacter* strains. No *Sinomonas* strain possessed *nifH* gene and siderophore producing activity (Fig. 10a). Only 1 out of 38 *Arthrobacter* strains and 10 out of 48 *Bacillus* strains could tolerate high salt concentration (8% NaCl) vs. 8 out of 11 *Staphylococcus* strains.

Neither *Leifsonia* nor *Sinomonas* strains showed tolerance to 8% of NaCl (Fig. 10b). Likewise, except *Arthrobacter* and *Bacillus* strains, no isolate belonging to other genera was able to solubilize Moroccan rock phosphate (Fig. 10c). The percentage of strains displaying two to three functional traits tested was about 78% in *Staphylococcus*, 67% in *Arthrobacter*, 63% in *Paenibacillus*, 57% in *Micrococcus*, 54% in *Bacillus* and 50% in *Leifsonia* strains. No genera possessed a strain displaying all the four traits tested. Interestingly, all *Arthrobacter, Paenibacillus, Sinomonas and Staphylococcus* strains displayed at least one functional trait (Fig. 10d).

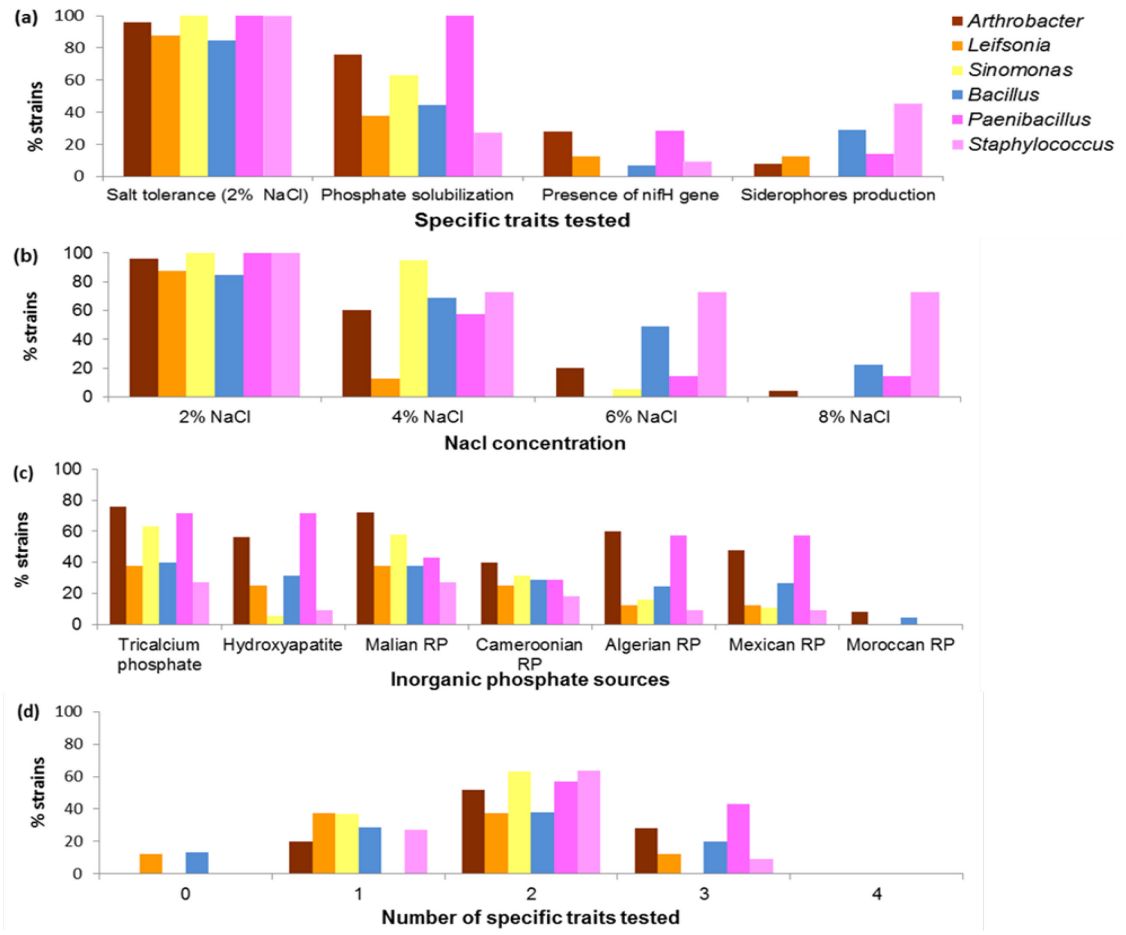


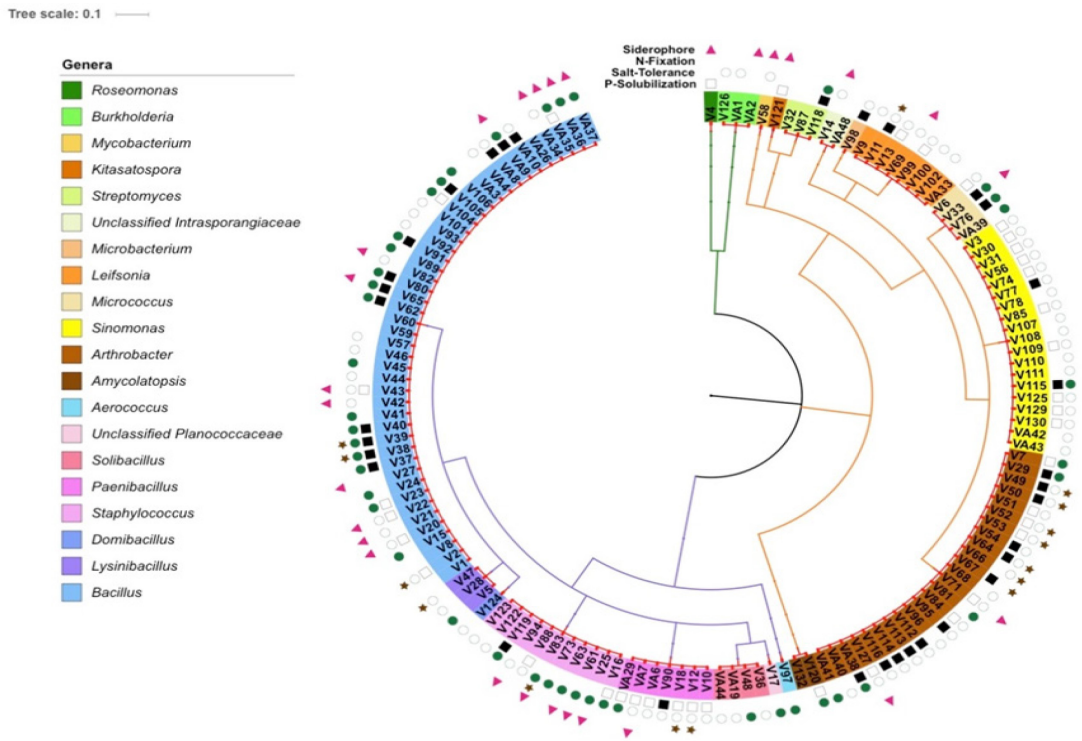
Figure 10. Percentage of isolates of the six most abundant genera displaying the different traits tested: *Arthrobacter* (maroon bars), *Leifsonia* (orange bars), *Sinomonas* (yellow bars), *Bacillus* (blue bars), *Paenibacillus* (fuchsia bars), *Staphylococcus* (light pink bars); (a) percentage of bacterial strains displaying salinity tolerance and (PGP) traits within each genus, (b) percentage of bacterial strains showing tolerance to different concentrations of NaCl within each genus, (c) percentage of bacterial strains solubilizing different inorganic phosphate compounds within each genus; RP = rock phosphate, (d) percentage of bacterial strains showing certain numbers of specific traits tested (from 0 = no trait, to 4 = all tested traits detected) within each genus.

We evaluated isolates individually for their ability and efficiency in expressing each functional trait (Fig. 11a) and to solubilize each inorganic phosphate (Fig. 11b) in relationship to their phylogenetic affiliation. Interestingly, no bacterial isolate, over all detected phyla, exhibited all the four specific traits tested in this study. However, a large number of strains were able to express multiple functional potentialities. Those displaying three traits were V2, V20, V22, V38, V39, V43, V65, VA9 and VA 35 belonging to *Bacillus*, V49, V50, V52, V53, V64, V71 and V127 belonging to *Arthrobacter*, three to *Paenibacillus* (V12, V18 and VA7), and one to *Staphylococcus* (V83), *Micrococcus* (Sahay and Patra, 2014) and *Leifsonia* (Sahay and Patra, 2014). For phosphate solubilization, only three bacterial isolates, the *Bacillus* strains V62 and V91, and the *Arthrobacter* strain V54, showed the ability to solubilize all seven inorganic phosphate sources tested (11b). In general, bacterial isolates exhibiting two to three abilities

concurrently were mostly represented by strains belonging to *Bacillus* (26/143) and *Arthrobacter* (20/143) genera.

Based on the above results, we chose six strains, two *Arthrobacter* (V54 and V64) and two *Bacillus* (V39 and V62) with high PGP and salinity tolerance potential, as well as V84 *(Arthrobacter* strain) and V1 (*Bacillus* strain) with less or no PGP and salinity tolerance potential, for further *in vivo* plant experiments. Among the selected isolates, V54 was the only *Arthrobacter* strain solubilizing all seven phosphate sources tested. V64 was closely related to V54, solubilizing six phosphate sources and additionally is a potential N_2-fixing strain. We randomly choose V84, not closely related to the two others, as a less efficient strain. Regarding *Bacillus* strains, V62 was selected as one of the two strains solubilizing all the seven phosphate sources. V39 was one of the two isolates (V39 and V38) displaying high phosphate solubilizing ability and additionally is a potential N_2-fixing strain. We arbitrarily selected V1, which is not closely related to others among the isolates without any trait.

(a) Different functional traits



(b) Phosphate solubilizing ability

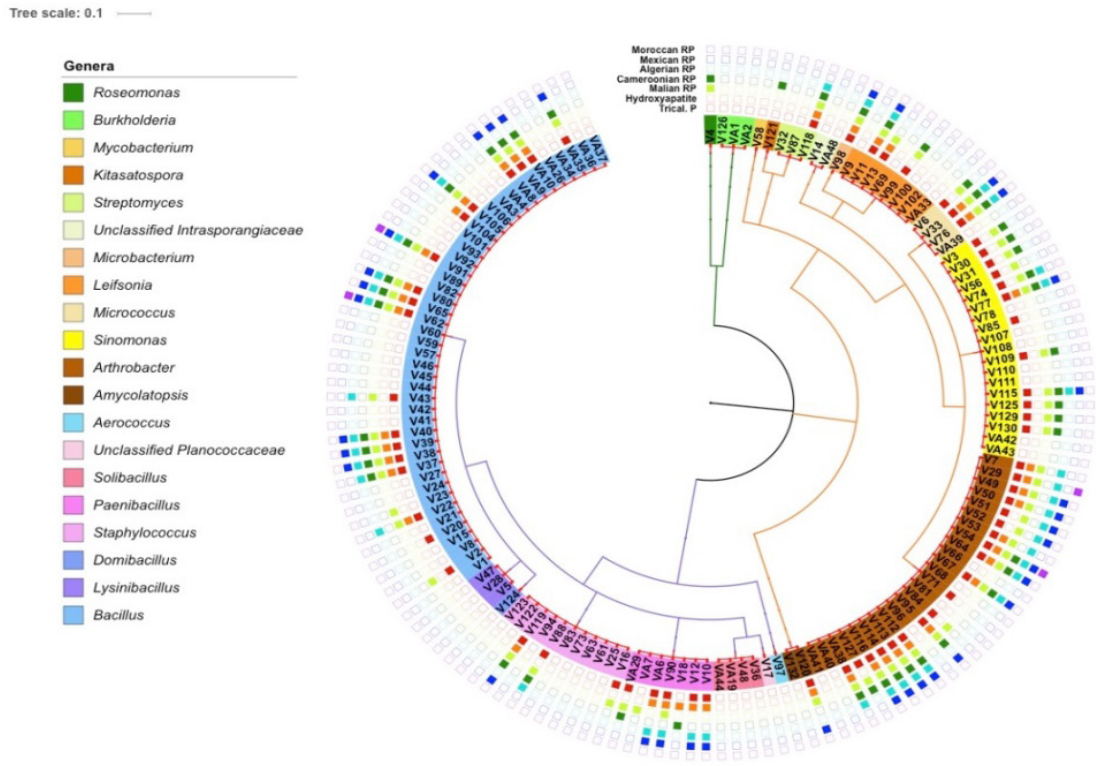


Figure 11. Bacterial isolates associated with the maize rhizophere displaying specific traits tested according to their taxonomic affiliation: (a) ability of each bacterial isolate in expressing salinity tolerance, phosphate solubilizing, *nifH* gene presence, and siderophore production activities. Phosphate solubilization: 1-4 phosphates sources (□), 5-7 phosphate sources (■), salt tolerance: 2-4% NaCl (○), 6-8% NaCl (●); detection of *nifH* gene responsible for N_2-fixation: presence (★); siderophore production: presence (▲); if unfilled, no activity. (b) Ability of each bacterial isolate to solubilize different phosphate sources. Positive: trical. P (■), hydroxyapatite (■), Malian RP (■), Cameroonian RP (■), Algerian RP (■), Mexican RP (■) and Morroccan RP (■); if unfilled, it is not able to solubilize; trical. P = tricalcium phosphate and RP = rock phosphate. The Interactive Tree of Life web-based tool was used to display the different traits that each bacterial isolate harbors on the trees.

3.4.3. Arthrobacter sp. and Bacillus sp. from maize rhizosphere accelerate seed germination

Selected bacterial strains, V84, V54 and V64 (*Arthrobacter* sp.) and V1, V62 and V39 (*Bacillus* sp.), were inoculated and tested against non-inoculated controls to evaluate their potential as PGPR in maize at the germination stage. Results clearly showed that these six bacterial strains had variable effects on hypocotyl and root lengths as well as the germination rate of maize seeds (Fig. 12). Bacterial inoculation significantly enhanced both hypocotyl and root lengths ($p \leq 0.05$) except for the effect of V1 (*Bacillus* strain) on hypocotyl length (Fig. 12c, 12d). No significant difference in the germination rate was observed compared to the control (Table 6). However, except for V84 *(Arthrobacter* strain), all bacterial treatments supported a germination rate superior or equal to the control.

Finally, a total of three tested bacterial isolates, two *Bacilli* (V39 and V62) and one *Arthrobacter* (V54), had consistently significant positive effects on the vigor index of maize seeds (Fig. 12b), Bacterial strains V64 and V39 with an increase of 44.5% and 41.1% were the most effective inoculants for hypocotyl length, while the best root growth was induced by V39 (120.4%) and V54 (118.3%) (Fig.12c; 12d). The best vigor index was obtained for seeds inoculated with V54, V39 and V62, producing an increase of 114.5%, 109.3%, and 100.7%, respectively. In all cases, V1 had the lowest effect compared to the other isolates tested.

Table 6: Effect of different bacterial treatment on maize seeds germination.

Treatments	% germination	Standard deviation
Control	88.889 (b)	9.6
V54	100 (b)	0
V64	88.889 (b)	9.556
V84	70.368 (a)	19.2
V62	94.444 (b)	9.6
V39	94.444 (b)	9.6
V1	88.889 (b)	9.6

The different letters indicate significant differences between treatments within a specific stress ($p \leq 0.05$) using the Tukey HSD test. Control (non-inoculated), V54, V64 and V84 (*Arthrobacter* sp.), V62, V39 and V1 (*Bacillus* sp.).

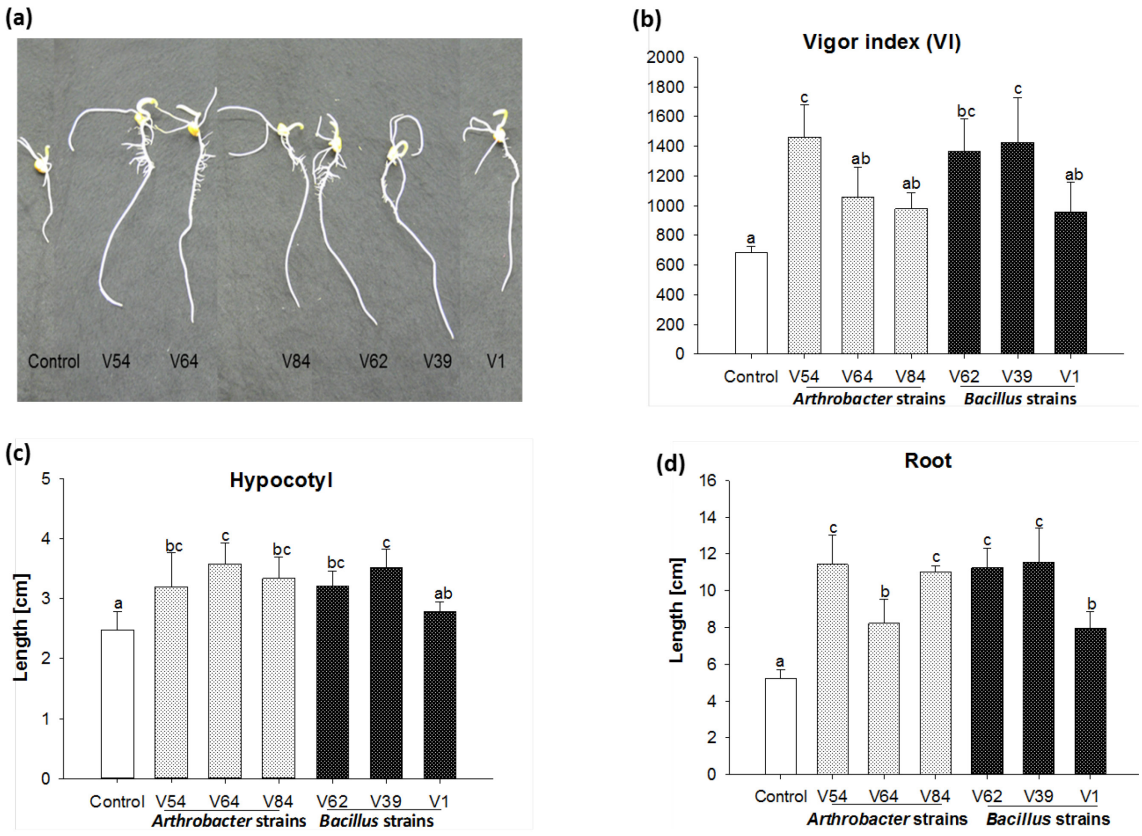


Figure 12. Effect of selected bacterial isolates on maize seedlings: (a) seed germination assay, (b) vigor index, (c) hypocotyl length, (d) root length of 18 maize seeds *(Zea mays* L. var. LUIGI CS) either inoculated with 0.05 M NaCl buffer (control) or 10^{8} CFU mL^{-1} of bacterial inoculum V84 (*Arthrobacter sp*.), V54 (*Arthrobacter sp*.), V64 (*Arthrobacter sp*.), V1 (*Bacillus sp*.), V62 (*Bacillus sp*.) or V39 (*Bacillus sp*.). Different letters indicate significant difference between treatments ($p \leq 0.05$) using turkey test. CFU= colony forming unit.

3.5. Discussion

The benefit PGP bacteria exert to plant growth and yield is well known. However, the growth-promoting effect depends mainly on native biotic and abiotic factors including bacterial species and the soil types. To improve the selection of climate and environmental adapted efficient PGP bacteria from Cameroonian soil, we used a combination of molecular/ bioinformatics tools and *in vitro* studies.

Partial 16S rDNA sequencing analysis assigned our isolates to 20 genera, belonging to 13 families categorized in three phyla: *Actinobacteria, Firmicutes and Proteobacteria*. The prevalence of bacteria, and members of these three phyla has already been observed in the rhizosphere of maize plants cultivated in a Mediterranean carbonate-rich soil (pH 8.5) in Spain and in different areas of the Rio Grande do Saul State in Brazil (Arruda et al., 2014; Garcia-Salamanca et al., 2013). Although differences between the geographic localizations, climatic conditions and soil properties exist, these phyla appear to be common in maize rhizosphere. This suggests that these bacteria colonize maize rhizosphere irrespectively of the soil type or

geographic location and might be used as maize fertilizers worldwide. The total number of bacterial genera (20) found in this study was higher than that obtained in recent studies also based on culture-dependent methods, which recorded numbers of 8, 7 and 6 bacterial genera (Abiala et al., 2015; Montañez et al., 2012; Pereira et al., 2011). This reveals a high diversity in the bacterial community linked to maize crops in Cameroon.

More than 48% of our isolates from Cameroonian soil were members of *Bacillus* and *Arthrobacter* genera, which clearly indicate abundance among isolates in the present work that could be representative within these genera living in association with maize in the soil studied. The dominance of *Bacillus* and *Arthrobacter* in maize rhizosphere is comparable to that reported by numerous studies on the microbial community associated with maize in Argentina and in tropical soil of Brazil (Gomes et al., 2001; Pereira et al., 2011). Bacteria of these two genera are widely distributed in various ecological niches and are common in the rhizospheres of a variety of plants such as wheat, sugarcane, rice, apple, grapevine and signalgrass (*Bracharria* sp.) (dos Passos et al., 2014; Marasco et al., 2013; Mutai et al., 2017; Rodrigues et al., 2016; Upadhyay et al., 2009). Other genera we isolated from Cameroonian soil samples, such as *Burkholderia*, *Lysinibacillus*, *Microbacterium*, *Micrococcus*, *Paenibacillus* and *Staphylococcus*, were also frequently isolated from maize rhizosphere populations grown in Argentina (Lopez-Reyes et al., 2015; Pereira et al., 2011). Some less frequently isolated genera such as *Amycolatopsis*, *Sinomonas, Kitasatospora*, *Solibacillu*s and *Streptomyces* have been reported by recent studies in Italy, Pakistan, Brazil and China (Ahmad et al., 2013; Gomes et al., 2014; Oliveira et al., 2009; Pathan et al., 2015; Zhao et al., 2014). Interestingly, despite extensive work on the microorganism residents of maize, *Aerococcus*, *Leifsonia*, *Roseomonas* and *Domibacillus* have not been found previously as maize associated bacteria. For the first time, our results show the occurrence of these bacteria in the maize rhizosphere using culture-dependent methods. However, some work revealed the presence of strains of these genera, possessing PGP activities in the rhizosphere of seagrass, bitter gourd, tomato, tuber and root crop like radish, carrot and potato (Ahmad et al., 2016; Jose et al., 2014; Kalam et al., 2017; Yanxing et al., 2017), although they are less known as PGPR.

Phylogenetic analysis based on the 16S rRNA gene sequence could not differentiate between strains belonging to *Bacillus cereus* and *Bacillus thuringiensis* in the *Firmicutes* group, nor *Arthrobacter* and *Sinomonas* strains in the *Actinobacteria* group. Difficulties in discriminating between bacterial strains belonging to the *B. cereus* group (*B. anthracis, B. thuringiensis* and *B. cereus*) via 16S rDNA sequence analysis have also been reported recently by other groups (Abaid-Ullah et al., 2015). Moreover, some strains reported as *Arthrobacter atrocyaneus* (Yamada and Komagata, 1972) have been reclassified as *Sinomonas atrocyanea* (Zhou et al., 2009). This is in agreement with the fact that the accuracy of 16S rRNA gene sequencing is

often limited for identifying bacteria at the species or even genera level (Abaid-Ullah et al., 2015; Kumar et al., 2014).

In the present study, we found in the explored conditions that the total population of isolated bacteria from the rhizosphere of maize grown in Cameroon is 6.64 x 10^7 CFU per gram of soil. Our result confirm those found by a recent study reporting a population size range from 2 x 10^6 - 8.2 x 10^8 CFU per g of soil of indigenous bacteria isolated from maize rhizospheres in different regions of Pakistan (Zahid et al., 2015). In contrast, a bacterial population ranging from 1.5 × 10^4 - 8.5× 10^6 CFU per g of soil was obtained in the rhizosphere of avocado trees from different regions of southern California (Nadeem et al., 2012). This variability in bacterial population sizes may be related to the soil type, plant species, farming practices, and climatic factors, which are the main determinants of composition in bacterial communities (Cavaglieri et al., 2009; Lopez-Reyes et al., 2015; Schrey et al., 2015; Vieira and Nahas, 2005). However, due to the high number of species present, as well as to the fact that most bacteria are viable but not cultivable (Barriuso et al., 2008), microorganisms that can be cultured in laboratory conditions occupied only a small fraction of these populations (<1%) (Yang et al., 2017). Thus, several authors used molecular techniques (Illumina pyrosequencing and molecular fingerprinting techniques) to decipher the dynamics and complexity of microbiota associated with plants (Correa-Galeote et al., 2016; Pereira et al., 2011; Yang et al., 2017). Nevertheless, the isolation and purification of beneficial bacterial strains remains essential in order to integrate knowledge about rhizosphere-associated bacterial communities obtained through molecular studies with data on their functional properties. This perspective opens new ways for targeted management of beneficial bacteria in sustainable food production systems (Battini et al., 2016).

Several studies have reported plant growth promotion and protection of PGPR by using bacteria possessing traits such as phytohormones production, pathogen suppression, ethylene production suppression, and heavy metal detoxifying potential (Abiala et al., 2015; Ahemad and Kibret, 2014; Ji et al., 2014; Nadeem et al., 2012; Ribeiro and Cardoso, 2012). However, our objective was to identify bacterial strains able to improve maize productivity under the nutrient deficient and saline conditions found in Cameroonian soils; this was the basis for selecting the different functional traits tested in this study. The potential of the isolated bacterial strains to tolerate salt and promote plant growth was first assessed *in vitro*. The *in vitro* screening for different traits is considered an effective tool for investigating microorganisms that can be used as bio-fertilizers. These tests are extremely important because they allow the selection of microorganisms with better agronomic potential before testing them *in planta* (Rodrigues et al., 2016; Szilagyi-Zecchin et al., 2014). An interesting feature of the bacteria studied here was their ability to tolerate salt.

Although direct plant growth-promoting mechanisms are highly important when selecting a potential strain for a biological preparation, the presence of traits such as resistance to abiotic stress helps the microorganisms to establish in the plant (Hayat et al., 2010; Rodrigues et al., 2016). Like in many soils of arid and semiarid areas, the problem of soil nutrient deficiency is generally associated with salinity in Cameroonian soils. Consequently, even a bacterial strain harboring high PGP abilities will not be able to colonize a plant root and promote plant growth if it cannot propagate in a saline environment. About 17% of the isolates were found to tolerate up to 8% NaCl, predominantly *Staphylococcus* and *Bacillus* strains. Other studies confirmed the high salinity tolerance of *Staphylococcus* (Parfentjev and Catelli, 1964; Tsai et al., 2011) and *Bacillus* (Nadeem et al., 2012; Siddikee et al., 2011) species. The halotolerant bacteria are able to withstand high salt concentrations because of their capability to accumulate compatible osmolytes to maintain intracellular osmotic balance. Use of halophilic or salt tolerant PGPR is an effective approach that has been employed successfully in various crops to improve their growth and tolerance under salt stress condition (Sharma et al., 2016). Tolerance to high salt concentrations could serve as a criterion for selecting strains for soil inoculation to improve crop salinity tolerance and crop production in Cameroon.

The capacity to solubilize different inorganic phosphate compounds is a promising attribute for selecting bacteria capable of increasing P availability in the rhizosphere. It is assumed that 1-50% of bacteria isolated from soil have the ability to solubilize P (Sharma et al., 2013). Mutai et al. (2017) analyzing the community of bacteria associated with *Brachiaria* grasses in Kenya, isolated 84 bacterial isolates in total, of which 56% were phosphate solubilizers. In agreement with these previous surveys, phosphate solubilizing activity was observed in 72 (50.34%) isolates regardless of the type of phosphates, although tricalcium phosphate was the one solubilized best. It is well known that tricalcium phosphate is more soluble in water than other hardly soluble phosphate sources, and it is commonly used as a universal factor for selecting phosphate solubilizing microorganisms. However, many isolates tested positive for solubilizing tricalcium phosphate fail when they are further tested for directly contributing to phosphorus nutrition in the plant. Therefore, multiple sources of insoluble phosphates are recommended for selecting efficient phosphate solubilizing bacteria (Sharma et al., 2013). Gomes et al. (2014) identified *Bacillus* and *Burkholderia* isolates as the most efficient to solubilize two natural phosphates extracted from Brazil mines. Some of our isolates belonging to *Arthrobacter* and *Bacillus* spp. were able to solubilize all the seven inorganic phosphate compounds tested, but interestingly, none of our isolated *Burkholderia* strains could solubilize any phosphate source. Phosphate solubilizing ability clearly depends on the phosphate source used.

With other direct PGP traits tested, about ten percent of bacterial strains isolated in this study revealed the 350 bp amplicon of the N_2-fixing marker gene *nifH*, predominantly *Arthrobacter*

strains. Such data confirm previous findings reporting the detection of the *nifH* gene in only two *Arthrobacter* strains among bacteria isolated from the wheat rhizosphere in India (Upadhyay et al., 2009). So, the existence of the *nifH* gene is used as an indirect evidence for a potential nitrogenase activity of these strains. The capability to fix atmospheric nitrogen is widespread among many bacterial genera such as *Bacillus*, *Burkholderia*, *Pantoea*, *Enterobacter* and *Erwinia*, isolated from the maize rhizosphere (Montañez et al., 2012; Zahid et al., 2015). By mediating the acquisition of nitrogen from the air and delivering it to the plant, these bacteria may be used as bio-fertilizers to improve crop productivity and reduce synthetic nitrogen fertilizer application (Ribeiro and Cardoso, 2012).

Siderophores are low molecular weight iron-chelating agents secreted by bacteria under iron-limiting conditions to help them scavenge iron from the environment (Neilands, 1981). Microorganisms producing siderophore can complex Fe^{3+} ions and stimulate plant growth by depriving plant pathogens of iron, which inhibits pathogen growth, and also by making iron available to the plants. More than half (Singh and Jha, 2016) of the bacteria isolated from Kenyan soil tested positive for siderophore production (Mutai et al., 2017). However, in our study, out of 143 isolates only 28 (19.58%) could produce siderophore. We found in the *Bacillus* group that some bacterial exhibiting the same functional traits, clustered together in the phylogenetic tree. For instance, bacterial strains with the highest phosphate solubilizing potential (V62 and V91) belong to the same clade of *Bacillus megaterium*. Likewise, those with high salinity tolerance, high phosphate solubilizing potential and N_2-fixing ability (V38 and V39) formed the clade of *Bacillus pumilus*. Therefore, our observations support the fact that closely related species often possess similar ecological features and functional capabilities (Morrissey et al., 2016).

We observed that rhizobacteria isolated in this study display multiple traits. We also noted a large variation among isolates of different genera with respect to the different combinations of traits they carried. About 15% of isolates expressed more than two of the studied traits, mostly belong to *Bacillus*, *Arthrobacter* and *Paenibacillus* genera. Other researchers have likewise reported that indigenous rhizobacteria commonly possess a variety of functional traits, alone or in combination, including N_2 fixation, phosphorus solubilization, siderophore production, and salinity tolerance (Ji et al., 2014; Montañez et al., 2012; Nadeem et al., 2012; Rodrigues et al., 2016; Silva et al., 2016; Upadhyay et al., 2009). Multiple traits are expected to be an advantage for seedling growth under multiple types of adverse conditions (Abiala et al., 2015). Indeed, upon inoculating six bacteria displaying different combinations of tested traits we saw much greater maize seedling growth stimulation when different traits were present in one strain.

It has previously been shown that indigenous Cameroonian bacterial isolates from palm tree rhizospheres in Cameroon also improved plant growth (Fankem et al., 2014). Although bacterial inoculation did not significantly increase the germination rate in our study, three strains, mainly the multiple PGP ones, significantly enhanced the hypocotyl and root length as well as vigor index of maize seeds compared to the non-inoculated control. This finding is consistent with a recent study reporting that maize growth was caused by a sum of factors and not by individual values obtained *in vitro*, thus suggesting the application of microorganisms possessing different growth promotion factors for *in vivo* plant tests (Rodrigues et al., 2016). However, the net effects of the simultaneous expression of different combinations of different traits on plant growth are still not well understood, and need to be elucidated under *in vivo* conditions (Baez-Rogelio et al., 2017).

3.6. Conclusion

For the first time we provide a comprehensive phylogenetic affiliation of cultivable bacterial communities associated with maize grown in Cameroon in relationship to their potential plant growth-promoting abilities. Our findings confirm our hypothesis that the rhizosphere of maize grown in Cameroon harbors a high diversity of cultivable bacteria exhibiting multiple plant growth-promoting and salinity tolerance activities, ideal for seedling establishment and growth. The findings demonstrate the potential of selected indigenous bacteria from Cameroon soil to enhance maize growth and productivity. Our approach using *in silico* selection procedures provides a time saving and cost efficient method to detect such bacteria. Further work will focus on the impact of simultaneous expression of different functional traits on plant growth under specific local conditions. Developing such microbial-based, low input tools may enhance the sustainability of crop production in sub-Saharan Africa.

CHAPTER 4: SELECTED RHIZOSPHERE BACTERIA HELP TOMATO PLANTS COPE WITH COMBINED PHOSPHORUS AND SALT STRESSES

This chapter is based on a manuscript: G. V. Tchuisseu Tchakounté, B. Berger, S. Patz, M. Becker, V. D. Taffouo, H. Fankem, S. Ruppel (2020). Selected rhizosphere bacterial strains help tomato plant cope with phosphorus and salt stresses. *Microorganisms, 8*, 1844.

4.1. Abstract

Plants are often challenged by multiple abiotic stresses simultaneously. The inoculation of beneficial bacteria is known to enhance plant growth under these stresses, such as phosphorus (P) starvation or salt stress. Here, for the first time, we assessed the efficiency of selected beneficial bacterial strains in improving tomato plant to better cope with double stresses in salty and P deficient conditions. Six strains of *Arthrobacter* and *Bacillus*, the most dominant genera of the rhizosphere in acidic soil in Cameroon, revealing different plant growth-promoting (PGP) traits were tested *in vitro* for their abilities to tolerate 2-8% (w/v) NaCl concentrations, and retain their motility and phosphate-solubilizing capacity under salt stress conditions. Whether these selected bacteria actually promote tomato plant growth under combined P and salt stresses was investigated in greenhouse experiments. Bacterial isolates from Cameroonian acidic soils mobilized P from different phosphate sources in shaking culture under both non-saline and saline conditions. They also enhanced plant growth in P deficient and salt-affected soils, and their PGP effect was even increased in higher salt stress conditions. The results provide valuable information for prospective production of effective bio-fertilizers based on the combined application of local rock phosphate and halotolerant phosphate-solubilizing bacteria. This constitutes a promising strategy to improve plant growth in P deficient and salt affected soils.

4.2. Introduction

An ever increasing human population, especially in developing countries, leads to the pressing needs to provide food security to upcoming generations (Yadav et al., 2017). Therefore, improving yield and sustaining soil fertility are of major interest to agricultural production worldwide. P fixation is a severe issue in Cameroon, where soils are predominantly acidic, characterized by high levels of exchangeable iron and aluminum, and extremely P deficient (Fankem et al., 2014; Tchuisseu Tchakounté et al., 2018). This problem is exacerbated by salinity, which is increasing in many agricultural soils, especially in semi-arid and arid regions where agriculture performs under irrigation (Srinivasan et al., 2012). Salinity negatively affects almost all aspects of plant development, including germination, vegetative growth and reproductive stages. It also suppresses P uptake via plant roots (Soni et al., 2013). Phosphorus deficiency in acidic and saline soils leads to the need for frequent applications of P nutrient.

However, expensive P fertilizers represent a major outlay for resource-poor farmers in developing countries like Cameroon. In addition to the financial cost, the excessive and non-coordinated application of inorganic fertilizers contributes to severe environmental problems such as groundwater contamination and waterway eutrophication (Alori et al., 2017; Kang et al., 2011).

Phosphorus availability can be enhanced by applying bacteria that exhibit phosphate-solubilizing activities as bio-inoculants. Different rhizosphere colonizing bacteria have been demonstrated to release organic phosphates or to solubilize inorganic phosphate compounds such as di and tricalcium phosphates, hydroxyapatite, and RPs (Oteino et al., 2015). Converting P into an accessible form is one of the important traits of plant growth-promoting rhizobacteria (PGPR). At present, powerful PSB identified primarily belong to the *Bacillus*, *Arthrobacter*, *Pseudomonas*, *Mycobacterium*, and *Enterobacter* genera, among others (Hanif et al., 2015; Rodriguez and Fraga, 1999; Wang et al., 2017). Phosphate-solubilizing activity of microorganisms is often decreased in saline soils (Cherif-Silini et al., 2013). Ability of microorganisms to adapt to P deficiency in salty soils is necessary for their growth and survival. Bacteria survival also depends on their motility capacity, a common trait among bacteria that confers a survival advantage in stressed environments. Motility allows bacteria to escape local stresses, translocate to a better nutritional condition in competitive environments, and efficiently invade host tissue (Harshey, 2003; Xie and Wu, 2014). In fact, bacterial surface motility is emerging as a major trait involved in many functions of plant-associated bacteria in regard to their ability to colonize and spread on/in their host (Venieraki et al., 2016). Therefore, seeking PSB possessing traits such as salt tolerance and high motility may open new perspectives for improving crop productivity in acidic and saline soils.

Members of the genera *Arthrobacter* and *Bacillu*s represent some of the more important soil bacteria, and are among plant PGPRs possessing many other PGP traits such as atmospheric nitrogen fixation, phytohormone synthesis, production of siderophores and plant pathogen suppression, besides salt tolerance and phosphate-solubilizing abilities (Upadhyay et al., 2009). Merging of multiple PGP traits in bacterial strains is expected to be advantageous for plant development under multiple types of adverse conditions. Several studies have underlined that stimulated plant growth by PGPR is rarely caused by a single bacterial activity, but is the net result of various mechanisms of action that are activated simultaneously. Thus, bacteria used *in vivo* should ideally exhibit different growth promotion factors for a greater effect. Increased plant growth and nutrient uptake have been reported in many crop species as a result of PGPR application under P starvation (Fankem et al., 2014; Oteino et al., 2015; Walpola and Yoon, 2013; Wang et al., 2017) or saline conditions (Egamberdieva et al., 2017a; Numan et al., 2018; Singh and Jha, 2016). However, studies on bacterial performance on plants exposed to concomitant P deficiency and salinity stress are still required. Until now, the majority of studies

have only focused on the *in vitro* characterization of potential bacteria that could be used to enhance plant growth under either P deficient or salt-affected soils.

A recent study reported the beneficial effect of PGPR inoculation on peanut growth in the presence of P and salt stresses (Jiang et al., 2018). Mitttler (2006) stated that a combination of stresses should be regarded as a new state of abiotic stress, since the response of each plant to bacterial inoculation under specific condition is unique and cannot be directly extrapolated from the corresponding response of plants to each stress applied individually (Mittler, 2006). Therefore, in this study we investigated the effect of six *Arthrobacter* and *Bacillus* strains, carrying different PGP traits for growth improvement and P uptake, on tomato plants under P starvation combined with different levels of salt stress. We assumed that bacteria able to solubilize P compounds *in vitro* even under saline conditions and which merge multiple PGP functional traits will improve tomato plant growth and P uptake, compared to strains of the same genera possessing fewer PGP traits. Our main goals were to: (i) evaluate the ability of the strains to solubilize various P sources *in vitro*; (ii) test *in vitro* the six bacterial strains for their ability to tolerate increasing NaCl concentrations, while retaining their motility; (iii) determine the bacterial phosphate-solubilizing activity under normal and salt stress conditions, and (iv) assess the bacterial inoculation effect on growth and P uptake of tomato plants under phosphorus stress combined with different levels of salt in a greenhouse.

4.3. Material and methods

4.3.1. Microorganism selection and storage

Three *Arthrobacter* strains (V54, V64 and V84) and three *Bacillus* strains (V62, V39 and V1) were used in this study. Bacterial strains were identified based on their partial 16S rRNA gene sequence as recently reported (Tchuisseu Tchakounté et al., 2018). Strains were stored at -80°C in standard nutrient broth medium with glycerol (50% v/v). The six strains were selected from a collection of 143 strains due to their different functional abilities when tested for their ability to tolerate salt, solubilize various phosphate sources, fix atmospheric nitrogen and produce siderophores (Tchuisseu Tchakounté et al., 2018).

4.3.2. Characterization of bacterial strains under normal condition

Motility test

To assess the swarming ability of bacterial strains, 10 µL of each bacterial suspension adjusted to OD 0.2 at 620 nm were spotted at the center of a plate containing 25 mL of a semisolid nutrient broth medium (NB; per liter: 5 g peptone , 3 g yeast extract and 5 g of Agar-Agar). For the swimming motility assay, the plate containing the nutrient broth medium (NB; per liter: 5 g peptone, 3 g yeast extract and 3 g of Agar-Agar) were inoculated with 10 µL of each bacterial suspension on the surface of the plate. For each bacterial strain, control plates were

maintained with nutrient broth (NB; per liter: 5 g peptone, 3 g yeast extract and 15 g of Agar-Agar). The plates in triplicate were incubated at 28°C for 48 h and motility (diameter of migration of the bacteria on top of the agar) was measured from the center towards the periphery of the plate.

Qualitative characterizations of bacteria for phosphate solubilization on plates

The efficacy of the different strains to solubilize sparingly soluble phosphate sources was first assessed on plates based on the halo zone formation and by using the solubilization index (SI) as an indicator for the strain efficiency (the higher the SI, the greater the solubilization ability; the solubilization index which equals 1 indicates the non-solubilization ability by the bacterial strain). The ability of the bacterial strains to solubilize all seven different inorganic phosphate sources (TCP, hydroxyapatite, Algerian RP, Cameroonian RP, Malian RP, Mexican RP and Moroccan RP) was assessed on plates filled with the National Botanical Research Institute's Phosphate growth medium (NBRIP; Nautiyal, 1999), as described already in (Chap 3). All RPs were analyzed for their chemical contents (Table 6). Plates in triplicate for each treatment and each phosphate source were incubated at 28°C for 5 days. The halo (yellow) zone surrounding the bacterial colony indicated phosphate solubilization. The solubilization index (SI) was used as an indicator for the isolate´s efficiency, and was calculated in the following way: SI= (colony diameter + diameter of halo zone) / colony diameter (Qureshi et al., 2012).

Quantitative estimation of phosphate solubilization in liquid broth

Bacterial strains were tested in liquid NBRIP media to assess their capability to release P from TCP and Cameroonian RP (CRP). The local RP source (CRP) as well as TCP, the most used inorganic phosphate, were chosen for screening phosphate-solubilizing microorganisms. In all cases, 50 mL NBRIP medium was distributed into 100 mL Erlenmeyer flasks. After sterilization and cooling, 1 mL of the bacterial suspensions was used to inoculate flasks. Each treatment was replicated three times and non-inoculated flasks supplemented with different phosphate sources and 1 mL of 0.05 M sterile NaCl served as controls. Incubation was performed at 28°C, 180 rpm for 5 days. At the end of the incubation time, the cultures were transferred into sterile falcon tubes, centrifuged at 10,000 g for 10 min at 4°C and the supernatants filtered through 0.2 µm filters. The pH of the filtrate was measured in each case and the available P was determined following the colorimetric molybdate blue method described by (Bae et al., 1999) and the pH of the supernatant was measured using pH 110 meter (VWR, Germany).

4.3.3. Characterization of bacterial strains under salt stress

Motility test under salt stress

The swarming and swimming ability of bacterial strains under salt stress was performed essentially as described above, on the same media supplemented with different concentrations of NaCl (2, 4 and 8%; w/v), and motility was measured as before.

Phosphate solubilization under salt stress

The influence of salt on the phosphate-solubilizing ability of the bacterial strains was performed essentially as described above, on NBRIP medium supplemented with different concentrations of NaCl (2 and 4%, w/v). Media inoculated with 1 mL of a fresh culture of each bacterial strain were incubated at 28 °C for 5 days. Cultures were centrifuged at 10,000 rpm for 10 min at 4°C. The amount of soluble phosphate was determined as described above.

4.3.4. Plant inoculation experiment

A greenhouse experiment using tomato plant (*Solanum lycopersicum* L., cultivar *Harzfeuer F1,* 94% germination rate) was conducted to evaluate the inoculation effect of the six bacterial strains: (*Arthrobacter* strains V54, V64 and V84; *Bacillus* strains V62, V39 and V1) on plant growth and P uptake under different growth conditions in the greenhouse. Phosphorus stress was triggered by fertilization with poorly soluble Cameroonian RP (CRP). The growth conditions included three treatments: P stress without salt stress (CRP + EC = 0 ds m^{-1}), P stress + low salt stress (CRP + low S; CRP + EC = 8 ds m^{-1}) and P stress + high salt stress (CRP + high S; CRP + EC = 12 ds m^{-1}). The inoculation comprised eight treatments including six bacterial treatments (inoculated plants with each one of the bacterial strains), a non-inoculated control (negative control) and a non-inoculated treatment fertilized with KH_2PO_4 (without phosphorus stress). Surface-sterilized tomato seeds were inoculated by immersion in 1 mL of each bacterial suspension (microbial treatments) or 1 mL 0.05 M sterilized NaCl solution (control treatments) for 15 min, then sown in quartz sand and maintained in a phyto-chamber (25/20°C day/ night temperature) for 14 days. Afterwards, seedlings were potted in pots containing 1 L of mixed quartz sand and vermiculite (1/1). All pots (CRP, CRP + low S and CRP + high S) were mixed with Cameroonian RP 350 mg P g^{-1} soil (equivalent to P fertilization of 80 kg P ha^{-1}). The positive control pots were supplemented with the same amount of P applied as soluble phosphate (KH_2PO_4). In all inoculated pots, seedlings were transferred to a pit and finely covered with soil and re-inoculated the following day with 2.5 mL of the respective bacterial suspension or with 0.05 M sterilized NaCl solution for the control treatments (negative and positive). Pots were watered with 30 mL of the corresponding modified, lacking P, Hoagland solution five times per week and with 30 mL osmose water twice per week. Salt stress was imposed by adding 20% NaCl to the Hoagland solution until reaching the targeted concentration. Plant growth was documented over six weeks after

transplanting in a greenhouse at a day/night temperature 25/23°C and 75% air humidity. The experimental design was a completely randomized block system with eight treatments and five replications for each treatment. At the harvest, the aerial part was separated from the root part and plant roots were thoroughly washed in tap water and deionized water. Shoot, root and total fresh biomass and shoot height were then recorded. The dry biomass of shoots and roots was determined after they were oven dried at 60°C for 72 h. Oven-dried tissues were used to determine the shoot and root P concentration as described in chapter 2 (2.16).

4.4. Results

4.4.1. Phosphate source and strain dependent phosphate-solubilizing efficacy on agar plates

Among the six tested bacteria, all strains, except *Bacillus* strain V1, were able to solubilize at least one inorganic phosphate source on the agar plates (Fig. 13). The phosphate-solubilizing ability across all P sources (TCP, hydroxyapatite, and Algerian, Cameroonian, Malian, Mexican and Moroccan RP) differed depending on the bacterial strain. *Arthrobacte*r strain V54 and *Bacillus* strain V62 were the only strains able to solubilize all the seven phosphate sources. *Bacillus* strains V62 and V39 showed the highest solubilization index (SI) for TCP: 5.1 and 5.1 respectively. Hydroxyapatite was best solubilized by V62 and V64 (SI = 4.0). V62 again displayed the highest SI with Algerian RP (3.9), Cameroonian RP (3.0), Mexican RP (5.0), and Moroccan RP (3.5), while V54 with the SI of 5.0 was the most efficient strain in solubilizing Malian RP (Fig. 13).

Regarding the performance of the different strains on plates, V62 can be considered as the most efficient strain over all the phosphate sources, followed by V54 and V39 (Fig. 14a). Regarding the average SI of all strains together, the phosphate sources were not equally solubilized: TCP (SI = 3.5) was the phosphate compound most easily solubilized by the bacterial strains, followed by Malian RP (SI = 3.4) and hydroxyapatite (SI = 2.8). Moroccan RP with a mean SI of 1.6 was the most recalcitrant phosphate source for the bacterial strains (Fig. 14b).

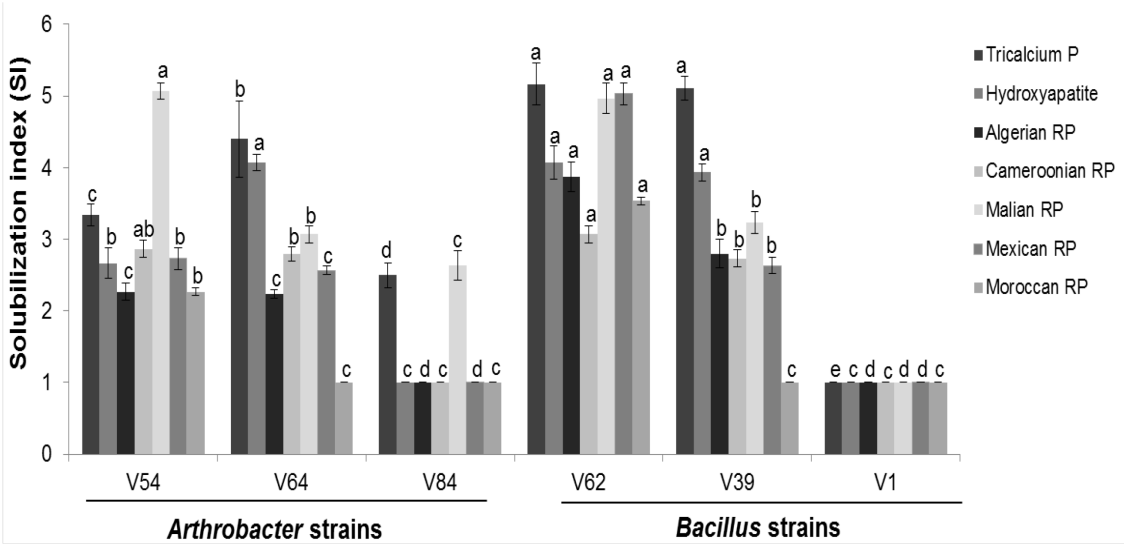


Figure 13. The efficiency of bacterial strains to solubilize inorganic phosphate sources on solid media. The solubilization index of three *Arthrobacter* (V54, V64, and V84) and three *Bacillus* (V62, V39, and V1) strains for the seven different inorganic phosphate sources, as measured on solid agar plates. The results are the means of three replicates. Error bars represent the standard deviation. Different letters indicate significant differences between bacterial strains within the same phosphate source ($p < 0.05$) using the Tukey HSD test. RP = rock phosphate

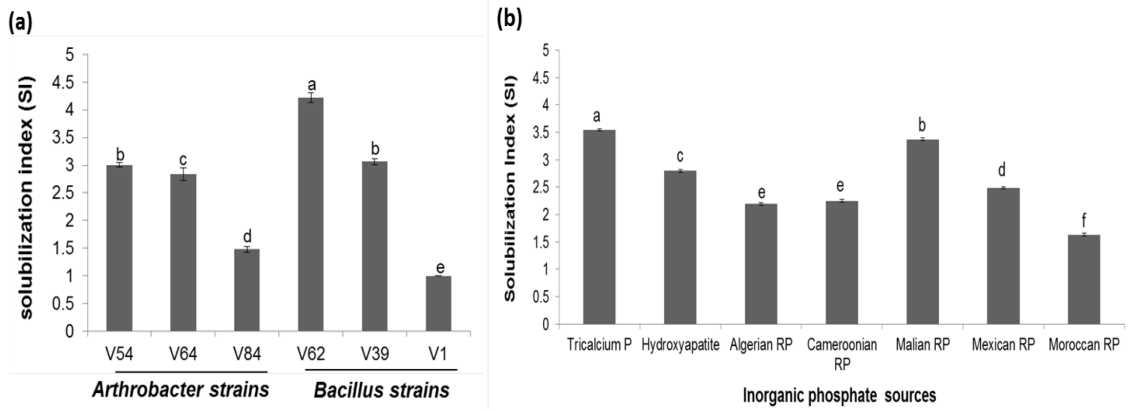


Figure 14. Phosphate solubilization on plate under normal conditions. (**a**) Mean activity of each bacterial strain in solubilizing all the seven inorganic phosphate sources, and (**b**) mean activity of all bacterial strains in solubilizing each individual inorganic phosphate source: tricalcium phosphate, hydroxyapatite, Algerian rock phosphate (RP), Cameroonian RP, Malian RP, Mexican RP and Moroccan RP. Data are means of three replicates for each treatment. Error bars represent the standard deviation and different letters indicate significant difference between strains and phosphate sources ($p < 0.05$) using the Tukey HSD test; RP = Rock phosphate.

4.4.2. Phosphate source and strain dependent phosphate-solubilizing efficiency in liquid culture under non-stress conditions

To quantify the specific bacterial phosphate-solubilizing activity in liquid culture, we used the most easily solubilized TCP and the local insoluble phosphate, Cameroonian RP (CRP). The amount of soluble P and changes in pH were monitored for five days in NBRIP medium. In the

liquid culture supplemented with TCP or CRP all bacterial strains solubilized phosphate, but at different rates, depending on the strain and the phosphate source. Bacterial inoculation significantly increased the TCP and CRP solubilization compared to the non-inoculated control (Tukey test $p < 0.05$; Fig. 15). Bacterial induced solved P amounts, estimated in the NBRIP supernatant, varied from 36.3 to 179.9 mg L^{-1} in TCP, with the highest solubilization recorded in *Arthrobacter* strains V84 (179.9 mg L^{-1}) and V54 (169.6 mg L^{-1}), followed by *Bacillus* strain V39 (127.1 mg L^{-1}). In contrast, with CRP, the estimated amount of P solubilized varied from 36.4 to 54.5 mg L^{-1} with the maximum concentration in *Bacillus* strain V62 (54.5 mg L^{-1}), followed by *Arthrobacter* strains V84 (44.2 mg L^{-1}) and V64 (42.9 mg L^{-1}). *Bacillus* strain V1, which was unable to show any phosphate-solubilizing activity on solid media supplied with the different phosphate sources, could mobilize phosphate from TCP and CRP in liquid culture. However, compared to all strains, it recorded the lowest P concentration of 36 mg L^{-1} with TCP and CRP.

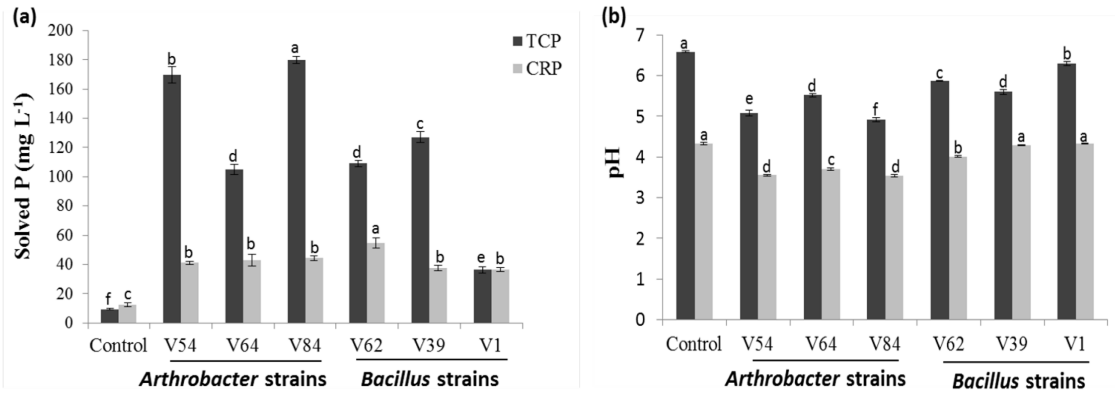


Figure 15. The efficiency of bacterial strains to solubilize inorganic phosphate sources in liquid culture. (**a**) Amount of P (mg L^{-1}) released from tricalcium phosphate (TCP) and Cameroonian RP (CRP). (**b**) Accompanying pH changes induced by isolates of NBRIP medium supplemented with the two phosphate sources without additional salt stress (right hand side). Data are the means of three replicates and error bars represent the standard deviation. The different letters indicate significant difference between bacterial strains ($p < 0.05$) using the Tukey HSD test. Control = NBRIP medium supplied with each of the phosphate source without bacterial inoculation. Control = NBRIP medium supplied either with TCP or CRP without bacterial inoculation.

Significant differences were observed between the mean activities of strains solubilizing the two different phosphate sources in liquid culture without additional salt stress. Unlike the results obtained on plates, *Arthrobacter* strain V84 seemed to be most effective in solubilizing the two phosphate sources in liquid culture (Fig. 16a). Regarding the average capability of all strains to solubilize each P source in liquid culture, as on solid media (shown above), TCP (105.1 mg L^{-1}) could be mobilized more easily than CRP (38.9 mg L^{-1}; Fig. 16b). Solubilization of phosphates was associated with a pH decrease in the NBRIP medium (Fig. 15b). This decrease was observed for all strains with the two phosphate sources. *Arthrobacter* strains were

more efficient in decreasing the pH than *Bacillus* strains, regardless of the phosphate source added to the medium. However, the drop in pH in the case of the NBRIP medium supplied with CRP was also observed in the non-inoculated control treatment. The result showed a negative correlation between the soluble P concentration and the pH (r = -0.9 and r = -0.5; $p < 0.05$) in TCP and CRP, respectively.

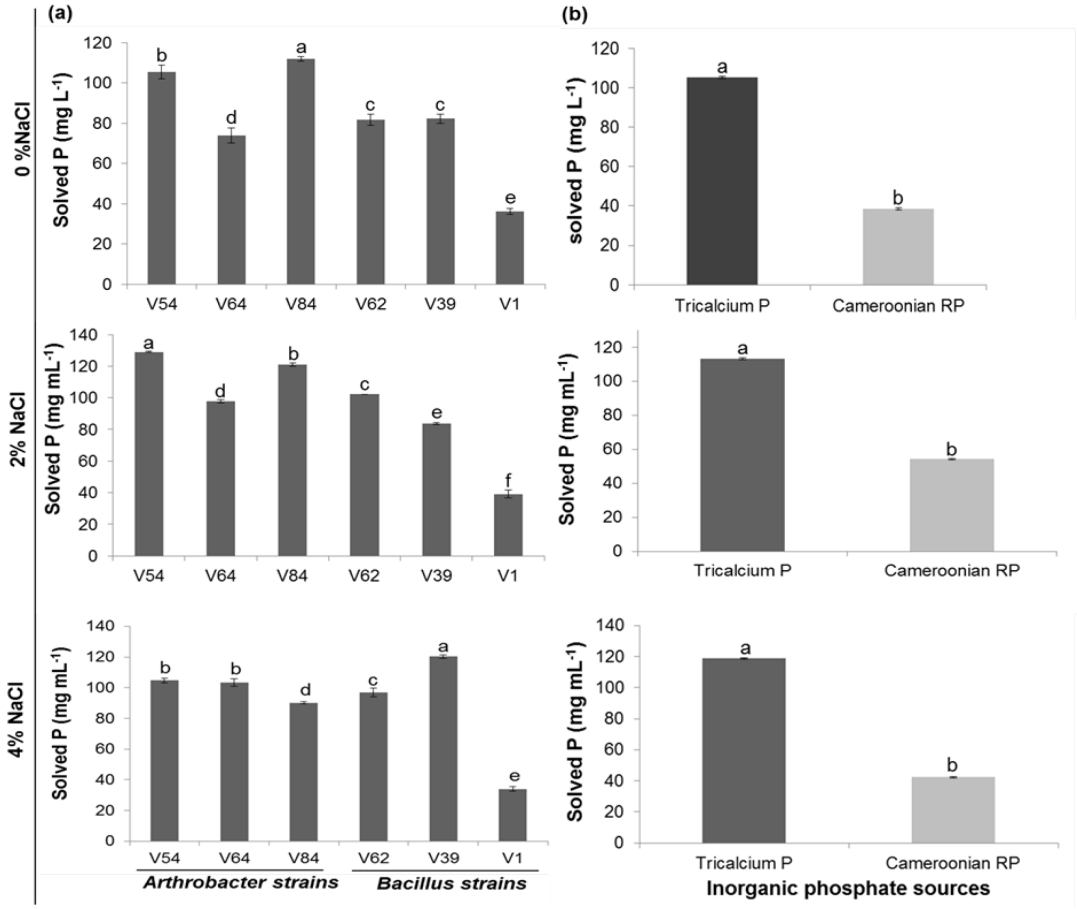


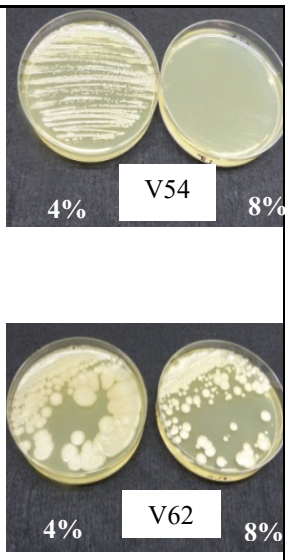
Figure 16. Phosphate solubilization at different NaCl concentrations in liquid culture. (**a**) Mean activity of each bacterial strain in solubilizing tricalcium phosphate and Cameroonian RP at 0%, 2% and 4% NaCl. (**b**) Mean activity of all bacterial strains to solubilize tricalcium phosphate and Cameroonian RP at 0%, 2% and 4% NaCl. Data are means of three replicates for each treatment. Error bars represent the standards deviation and different letters indicate significant difference between salt stress conditions within the same phosphate fertilization ($p < 0.05$) using the Tukey HSD test.

4.4.3. Bacillus strains tolerated higher salt than Arthrobacter strains and revealed best swarming and swimming abilities under salt stress conditions

All the bacterial strains were individually screened for their salt tolerance at graded concentrations of NaCl (2-16%, w/v). The results show a higher salt tolerance of the *Bacillus* strains, which even tolerated up to 10% NaCl (except V1), compared to the *Arthrobacter* strains used in this study (Table 1). All bacterial strains except V1 were able to tolerate at least 4% NaCl on agar plates.

Table 7: Salt tolerance of different bacterial strains

Bacterial isolates		Salinity tolerance (% NaCl)								
		0.05	2	4	6	8	10	12	14	16
Arthrobacter strains	V54	+	+	+	-	-	-	-	-	-
	V64	+	+	+	-	-	-	-	-	-
	V84	+	+	+	-	-	-	-	-	-
Bacillus strains	V62	+	+	+	+	+	+	-	-	-
	V39	+	+	+	+	+	+	+	+	-
	V1	+	+	-	-	-	-	-	-	-



(+) = growth; (-) = no growth

Bacterial swarming and swimming abilities differed depending on the strain and the salt (NaCl) concentrations (Table 2). High swarming and swimming abilities were observed in all bacterial strains at 0% NaCl (normal conditions). However, this ability of most bacterial strains decreased with increasing salt concentration. At 2% NaCl all strains showed at least a low swarming or swimming potential. The *Arthrobacter* strain V54 showed swarming and swimming potential up to 4% NaCl and *Bacillus* strains V62 and V39 even at respectively 10 % and 12 % NaCl.

Table 8: Swarming (0.5% agar) and swimming (0.3% agar) abilities of bacterial strains at different concentrations of NaCl

Bacterial isolates		0 % NaCl		2 % NaCl		4 % NaCl		8 % NaCl		10 % NaCl		12 % NaCl		14 % NaCl		16 % NaCl	
		0.5% agar	0.3% agar	0.5% agar	0.3% agar	0.5% agar	0.3% agar	0.5% agar	0.3% agar	0.5% agar	0.3% agar	0.5% agar	0.3% agar	0.5% agar	0.3% agar	0.5% agar	0.3% agar
Arthrobacter **strains**	**V54**	+++	+++	+++	+++	++	+++	-	-	-	-	-	-	-	-	-	-
	V64	+++	+++	+++	+++	++	++	-	-	-	-	-	-	-	-	-	-
	V84	+++	+++	++	++	++	++	-	-	-	-	-	-	-	-	-	-
Bacillus **strains**	**V62**	+++	+++	+++	+++	+++	+++	++	++	++	++	-	-	-	-	-	-
	V39	+++	+++	+++	+++	+++	+++	++	++	++	++	++	++	++	-	-	-
	V1	+++	+++	++	++	-	-	-	-	-	-	-	-	-	-	-	-

(-) = no motility; (++) = low motility; (+++) = high motility

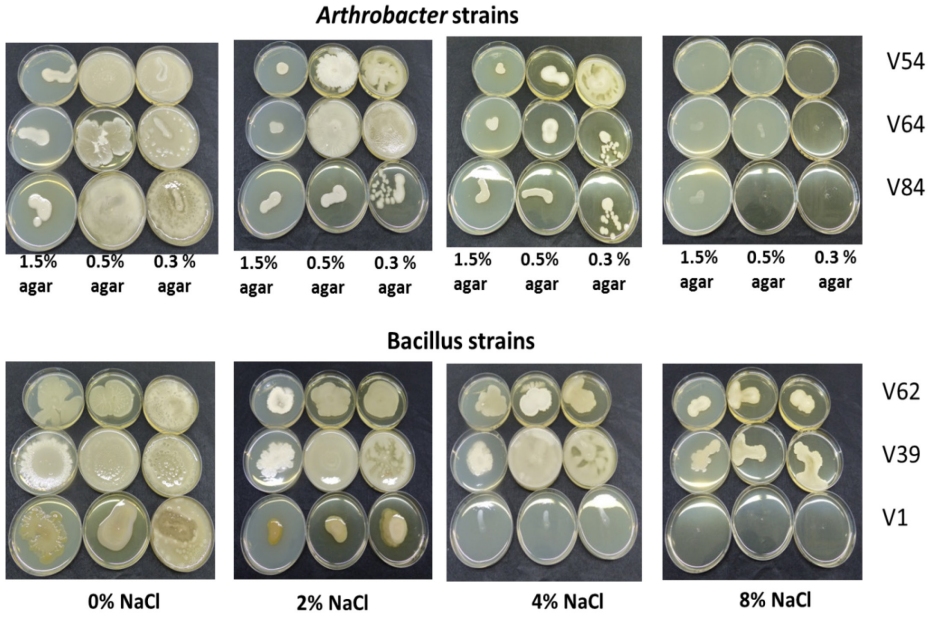


Figure 17. Swarming and swimming abilities of *Arthrobacter* and *Bacillus* trains at different concentrations of NaCl on plates.

4.4.4. Effect of phosphate source and salt on phosphate-solubilizing activity

To determine their phosphate-solubilizing activity under saline conditions, bacterial strains were also tested for their ability to solubilize TCP and CRP in the presence of different concentrations (2% and 4%) of NaCl in NBRIP broth (Table 9). The results were compared to the results obtained under normal condition (0% of NaCl) to determine the influence of salt on the amount of P released from TCP and CRP in NBRIP medium (Fig. 18).

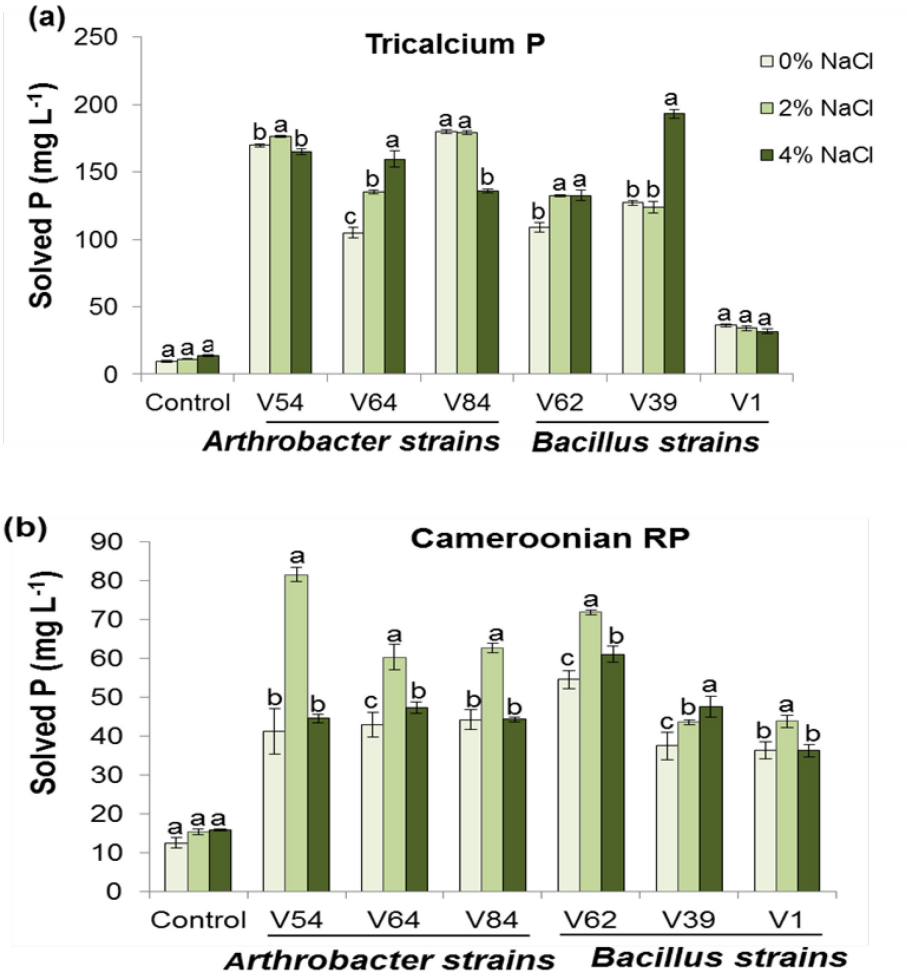


Figure 18. Effect of different NaCl concentrations on phosphate-solubilizing abilities of bacterial strains. (**a**) Amount of P released from tricalcium phosphate (TCP), and (**b**) amount of P (mg L^{-1}) released from Cameroonian RP (CRP) by the different bacterial strains at 0, 2 and 4% of NaCl. Results are the mean values of three replicates for each treatment. Error bars represent the standard deviation and different letters indicate significant salt effects for each strain separately ($p < 0.05$) using the Tukey HSD test. Control = NBRIP medium supplied either with TCP or CRP without bacterial inoculation.

Similar to normal conditions, the solubilizing capacity of the strains differ significantly ($p < 0.05$; Tukey HSD test) depending on the phosphate source and the salt concentration. In general, *Arthrobacter* strains were more efficient in solubilizing TCP in the presence of 2% of NaCl, with

the highest solubilization in V84 (179.2 mg P L^{-1}) and V54 (176.4 mg P L^{-1}). In contrast with CRP, the greatest amount of solubilized P at the same concentration of NaCl was found in *Arthrobacter* V54 (81.5 mg P L^{-1}) and *Bacillus* strain V62 (71.9 mg P L^{-1}). In the presence of 4% NaCl, the highest amount of solubilized P was recorded for *Bacillus* strain V39 (193.1 mg P L^{-1}) in TCP, followed by *Arthrobacter* strain V54 (164.9 mg P L^{-1}). *Bacillus* strains V62 (61.04 mg P L^{-1}) and V39 (47.5 mg P L^{-1}) showed the highest capability to release P from CRP (Table 9).

The analysis of variance also showed a significant effect of salt concentration ($p < 0.05$) on the phosphate solubilizing activity of strains. Unexpectedly, all bacterial strains expressed a higher phosphate solubilizing activity of CRP at 2% NaCl compared to normal growth conditions (Fig. 18b). *Bacillus* strain V39 showed an even higher phosphate-solubilizing activity when the NaCl concentration increased to 4%. The salt concentration effect varied specifically with the bacterial strain; however all selected bacterial strains solubilized at least the same amount of P from both phosphate sources (TCP and CRP) under 2% and even 4% NaCl as under normal growth conditions (Fig. 18a,b). The efficiency of solubilizing the P sources between strains under saline condition was also significantly different (Fig.16a). In the presence of 2% NaCl the highest solubilization efficiency for the two phosphate sources in liquid culture was observed for *Arthrobacter* V54 (128.3 mg P L^{-1}), followed by *Arthrobacter* V84 (120.3 mg P L^{-1}). At 4% NaCl, the highest efficiency of solubilizing the two phosphate sources was observed with *Bacillus* strain V39 (120 mg P L^{-1}), followed by *Arthrobacter* strains V54 (104.7 mg P L^{-1}) and V64 (103.3 mg P L^{-1}). Regarding the mean activity of all strains to solubilize each phosphate source, like normal conditions, was with TCP 113.2 mg P L^{-1} at 2%, and 118.8 mg P L^{-1} at 4% NaCl, the phosphate source most easily mobilized, and with CRP 54mg P L^{-1} at 2%, and 42 mg P L^{-1} at 4% NaCl, i.e. with the most recalcitrant phosphate compound (Fig. 16b).

Table 9: Efficiency of bacterial strains to solubilize inorganic phosphate at 2 % and 4 % NaCl in liquid culture: amount of P (mg L^{-1}) released from tricalcium P (TCP) and Cameroonian RP (CRP) and accompanying pH changes induced by bacterial isolates (NBRIP medium supplemented with the two phosphate sources and 2 % or 4 % NaCl).

Bacterial strains	2 % NaCl				4 % NaCl			
	TCP		CRP		TCP		CRP	
	Amount of released P (mg L^{-1})	pH	Amount of released P (mg L^{-1})	pH	Amount of released P (mg L^{-1})	pH	Amount of released P (mg L^{-1})	pH
Control	11.2 ± 0.30 ^{f}	6.7 ± 0.01 ^{a}	15.4 ± 0.70 ^{f}	4.5 ± 0.23 ^{a}	13.76 ± 0.53 ^{e}	6.6 ± 0.005 ^{a}	15.8± 0.30 ^{d}	4.5 ± 0.02 ^{a}
V54	176.4 ± 0.48 ^{a}	5.2 ± 0.01 ^{f}	81.5 ± 1.86 ^{a}	3.7 ± 0.02 ^{f}	164.9 ± 2.22 ^{b}	5.4 ± 0.02 ^{ef}	44.5 ± 1.03 ^{b}	4.1 ± 0.01 ^{c}
V64	135.2 ± 1.62 ^{bc}	5.6 ± 0.02 ^{d}	60.3 ± 3.17 ^{c}	3.8 ± 0.005 ^{ef}	159.5 ± 6.03 ^{b}	5.5 ± 0.01 ^{d}	47.2 ± 1.47 ^{b}	4.1 ± 0.02 ^{c}
V84	179.2 ± 1.16 ^{a}	5.2 ± 0.02 ^{e}	62.6 ± 1.28 ^{c}	3.8 ± 0.07 ^{e}	136.0 ± 1.54 ^{c}	5.4 ± 0.04 ^{f}	44.3 ± 0.60 ^{b}	3.9 ± 0.01 ^{d}
V62	132.5 ± 0.79 ^{c}	5.9 ± 0.01^{c}	71.9 ± 0.62 ^{b}	4.1 ± 0.02 ^{d}	132.6 ± 3.71 ^{c}	5.8 ± 0.01 ^{c}	61.0 ± 2.12 ^{a}	4.0 ± 0.1 ^{c}
V39	123.7 ± 4.37 ^{d}	5.9 ± 0.005 ^{c}	43.6 ± 0.62 ^{e}	4.5 ± 0.01 ^{b}	193.1 ± 3.16 ^{a}	5.5 ± 0.01 ^{de}	47.6 ± 2.67 ^{b}	4.3 ± 0.01 ^{b}
V1	34.2 ± 1.89 ^{e}	6.5 ± 0.02 ^{d}	43.7 ± 1.68 ^{d}	4.4 ± 0.01 ^{c}	31.9 ± 1.72 ^{d}	6.4 ± 0.01 ^{b}	36.3 ± 1.54 ^{c}	4.3 ± 0.01 ^{b}

Results are the mean values of three replicates for each treatment and the error bars represent the standard deviation. The different letters indicate significant differences between bacterial strains separately for each treatment (NaCl concentration and phosphate source) ($p < 0.05$) using the Tukey HSD test. TCP = tricalcium phosphate and CRP = Cameroonian RP.

Under saline conditions (2% and 4% NaCl) the solubilization of the two phosphate sources in liquid medium corresponded to a decrease in the medium pH (Table 9). This decrease was observed for all strains with the two phosphate sources. *Arthrobacter* strains were more efficient in decreasing the pH than *Bacillus* strains regardless the phosphate source added to the medium and irrespective of the salt concentration. Soluble P concentrations correlated negatively with the pH of the medium (TCP, r = -0.9; CRP, r = -0.8, p< 0.05) and (TCP, r = -0.9; CRP, r = -0.7, p< 0.05) at 2% and 4% NaCl, respectively.

4.4.5. Bacterial inoculations promoted tomato plant growth and P uptake even more efficiently under increased salt stress and P-deficient conditions

Verification of phosphorus deficiency and salinity effects on the growth and P uptake of tomato plants

To verify the effect of P deficiency and salt stress levels on the growth and P uptake of non-inoculated tomato plants, control plants with added Cameroonian RP and three levels of salt stress were compared to a non-stressed control fertilized with the easily accessible phosphate source (KH_2PO_4). The influence of P and salt stresses applications on plant height and shoot, root and total dry matter as well as P uptake were recorded (Fig.19 and Table 10).

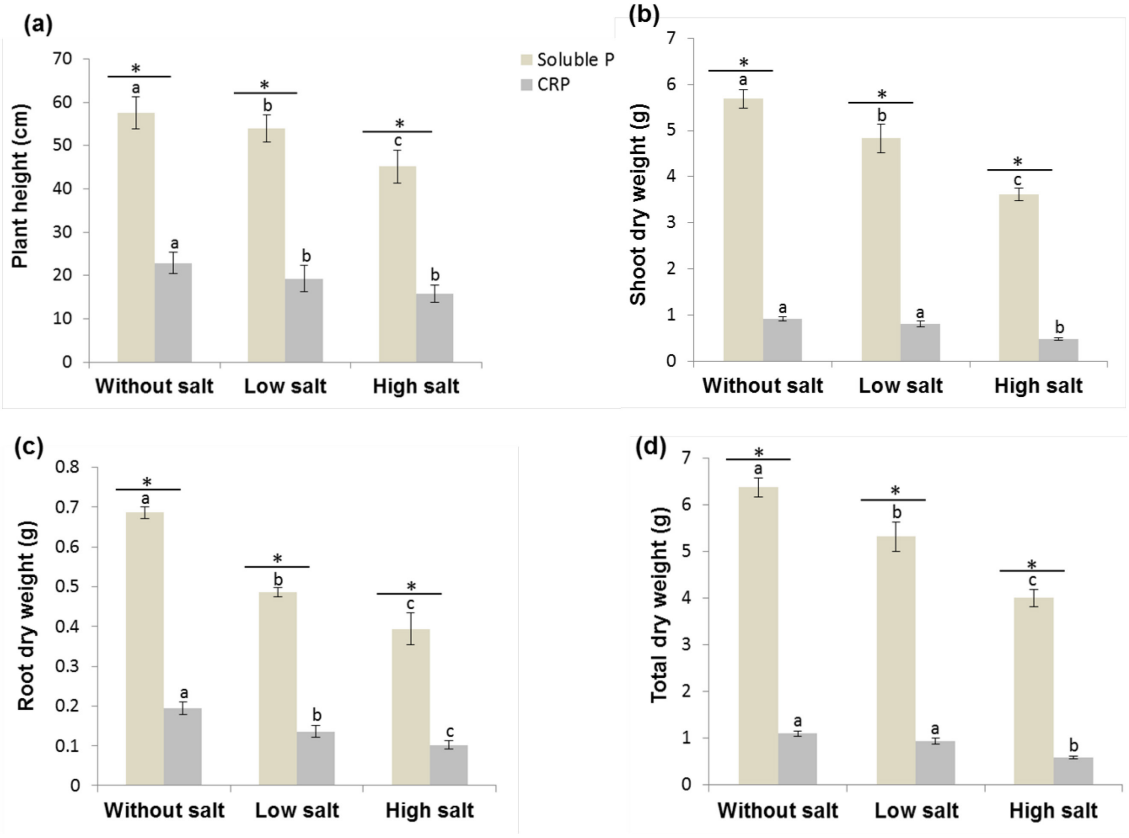


Figure 19. Effects of phosphorus stress and salinity on (**a**) plant height, (**b**) shoot, (**c**) root, and (**d**) total dry weights of control tomato plants (without bacterial inoculation) grown under different levels of salt stress: without, low salt stress (8 ds m^{-1}) and high salt stress. Beige bars represent plants supplied with soluble phosphate, KH_2PO_4, and grey bars plants supplied with hardly accessible phosphate (Cameroonian rock phosphate: CRP). Data shown are the mean of five replications and the error bars represent the standard deviation. Different letters indicate significant difference between salt stress conditions separately for each phosphate fertilization treatment and stars indicate significant difference between plants supplied with soluble phosphate and plants supplied with CRP under the same salt stress condition ($p < 0.05$) using the Tukey HSD test.

Salt addition, low salt stress (8 ds m^{-1}) and high salt stress significantly reduced plant growth, i.e. plant height and dry weights under easily available KH_2PO_4 fertilization (Fig. 19). P stress induced by fertilization with hardly accessible CRP significantly decreased plant height and dry

matter in tomato plants grown under salt stress regardless of salt stress levels (Fig. 19). For plants supplied with soluble phosphate (KH_2PO_4), no significant decrease in shoot, root and total P content was observed between plants grown under no salt stress and low salt stress (Table 10). Regarding plants supplied with hardly soluble CRP, the salinity effects on tomato plant growth were significantly different under high salt stress conditions. Except for plant height and root dry weight, the combined P deficiency and salt stress effects were not additive for other growth parameters in low salt stress (P stress and low salt stress). The values remained close to that of plants growing under no salt stress (P stress alone). Similarly, the effects of the two factors were not additive for shoot, root and total P uptake in CRP + high S (Table 10).

Table 10: Effects of P and salt stresses on P shoot and root contents and total P uptake of control tomato plants grown under different salt stress conditions

Plant organs	Growth conditions	Soluble P	Cameroonian RP (CRP)
P Shoot (mg shoot^{-1})	Without salt	55.2 ± 1.62 ^{a}	0.7 ± 0.08 ^{a}
	Low salt	53.2 ± 2.61 ^{a}	0.7 ± 0.04 ^{a}
	High salt	43.8 ± 1.82 ^{b}	0.4 ± 0.02 ^{a}
P Root (mg P root^{-1})	Without salt	2.8 ± 0.35 ^{a}	0.2 ± 0.01 ^{a}
	Low salt	2.7 ± 0.24 ^{a}	0.1 ± 0.02 ^{a}
	High salt	1.8 ± 0.10 ^{b}	0.1 ± 0.002 ^{a}
Total P (mg P plant^{-1})	Without salt	57.9 ± 1.36 ^{a}	0.9 ± 0.09 ^{a}
	Low salt	55.9 ± 2.67 ^{a}	0.8 ± 0.04 ^{a}
	High salt	45.5 ± 1.85 ^{b}	0.5 ± 0.02 ^{a}

Without salt = (0 ds m^{-1}), low salt stress = (8 ds m^{-1}) and high salt stress = (12 ds m^{-1}). Data are means of five replicates for each treatment. Different letters indicate significant difference between salt stress conditions within the same phosphate fertilization ($p < 0.05$) using the Tukey HSD test.

Selected bacterial strains promoted tomato plant height and dry mass production even under combined P and salt stress conditions

All six selected bacterial strains caused a significant increase in shoot dry weight compared to the non-inoculated control plant under P deficient and high salt stress conditions (CRP + high S, Fig. 21a). Among the six bacterial inoculations, two bacterial strains, V54 (*Arthrobacter* sp.) and V39 (*Bacillus* sp.), significantly enhanced (ANOVA, $p < 0.05$) plant height under CRP and CRP + high S; V54 alone induced a significant increase in plant height under CRP + low S. Strain V54 exhibited the greatest effect on plant height under CRP (24.1%) and CRP + low S (26.1%), whereas V39 promoted the highest plant height under CRP + high S (27.8 %; Fig.20).

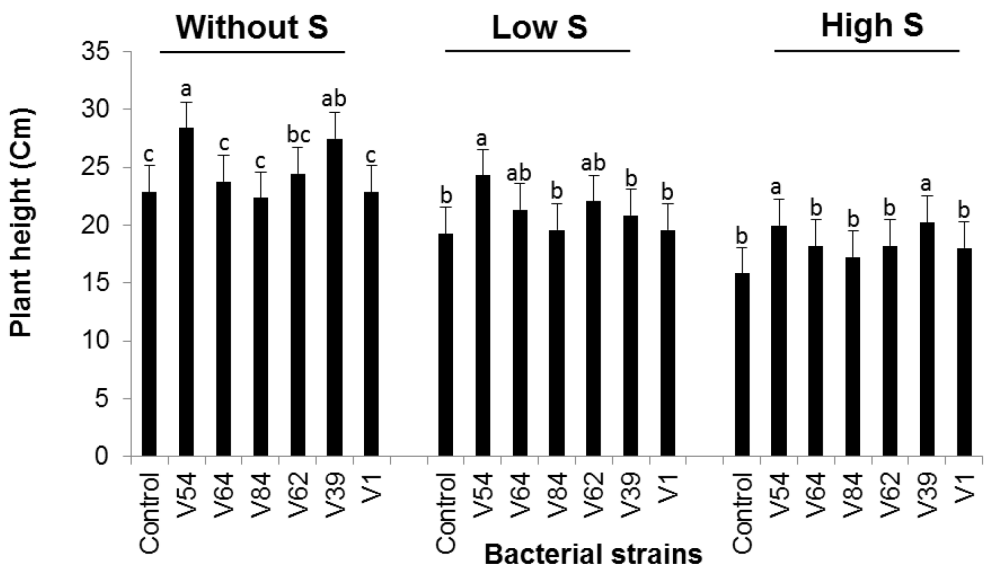


Figure 20. Effects of bacterial strains compared to the non-inoculated control on plant height of tomato plants grown under phosphorus stress combined to different level of salt stress (without salt (S); low S; high S), six weeks after planting in greenhouse. Data are means of five replicates for each treatment. Error represent the standards deviation and different letters indicate significant difference between salt stress conditions within the same phosphate fertilization ($p < 0.05$) using the Tukey HSD test. All plants were under phosphorus stress, amended with hardly accessible Cameroonian RP (CRP). Without S = phosphorus stress without salt stress, Low S = phosphorus stress + low salt stress, and High S = phosphorus stress + high salt stress. Control (non-inoculated), V54, V64 and V84 (*Arthrobacter* sp.), V62, V39 and V1 (*Bacillus* sp.).

In the same context, three strains (V39, V54 and V62) led to a significant increase (ANOVA, $p < 0.05$) in the shoot dry weight over the non-inoculated control respectively in CRP and CRP + low S. Tomato plants inoculated with *Bacillus* strain V39 showed the highest effect on shoot dry weight in CRP (109.6 %) and CRP + high S (122.6 %), whereas *Arthrobacter* strain V54 (50.6%) induced the biggest effect on shoot dry weight under CRP + low S. For root dry weight, three bacterial strains (V39, V54 and V64) promoted a significant increase over the negative control under CRP, and five strains, except *Arthrobacter* strain V84, significantly enhanced root dry weight compared to the negative control under CRP + low S and CRP + high S. Plants inoculated with V54 had the maximum effect on root dry weight under CRP (72.1%) and CRP + low S (73.5%), while V39 (80.3%) showed the highest effect on root dry weight under CRP + high S (Fig. 21b). With total dry biomass, the significant relative increase in CRP, CRP + low S and CRP + high S varied between 45-103%, 23-54% and 47-115%, respectively, compared to the non-inoculated control (Fig. 21c).

Effect of bacteria on P uptake of the tomato plant

Phosphorus shoot and root contents as a direct measure of PGP activities were clearly affected by different bacterial inoculants. Significant differences between treatments (ANOVA, $p <$

0.05) were found in the P content of the tomato plants. Three strains (V39, V54 and V62) under CRP, two strains (V54 and V62) under CRP + low S and all the strains under CRP + high S resulted in significant increases in P shoot concentration of tomato plants compared to the negative control (Fig. 21d). The maximum increase was recorded for plants inoculated by *Bacillus* strain V39 in CRP (1.8 mg shoot^{-1}), while *Arthrobacter* strain V54 induced the highest P shoot content under CRP + low S (1.1 mg shoot^{-1}) and CRP + high S (1.0 mg shoot^{-1}). Regarding the P root content, inoculation with two bacterial strains (V39 and V54) under CRP, one (V54) under CRP + low S and three (V39, V54 and V64) under CRP + high S resulted in a significant increase compared to the negative control. *Arthrobacter* strain V54 caused the highest increase under all growth conditions: (0.4 mg root^{-1}), (0.2 mg root^{-1}) and (0.2 mg root^{-1}) respectively under CRP, CRP + low S and CRP + high S (Fig. 21e). Similarly, the result of bacterial inoculation revealed that the highest amount of total P uptake was obtained in tomato plants inoculated with *Bacillus* strain V39 (2.0 mg plant^{-1}) and *Arthrobacter* strain V54 (2.0 mg plant^{-1}), with an increase of 122% and 118%, respectively, over the non-inoculated control plants under CRP. The maximum value of the total P uptake was achieved by inoculation with *Arthrobacter* strain V54 under CRP + low S (1.3 mg plant^{-1}) and CRP + high S (1.2 mg plant^{-1}), which induced an increase of 65% and 117%, respectively, over the non-inoculated control (Fig. 21f).

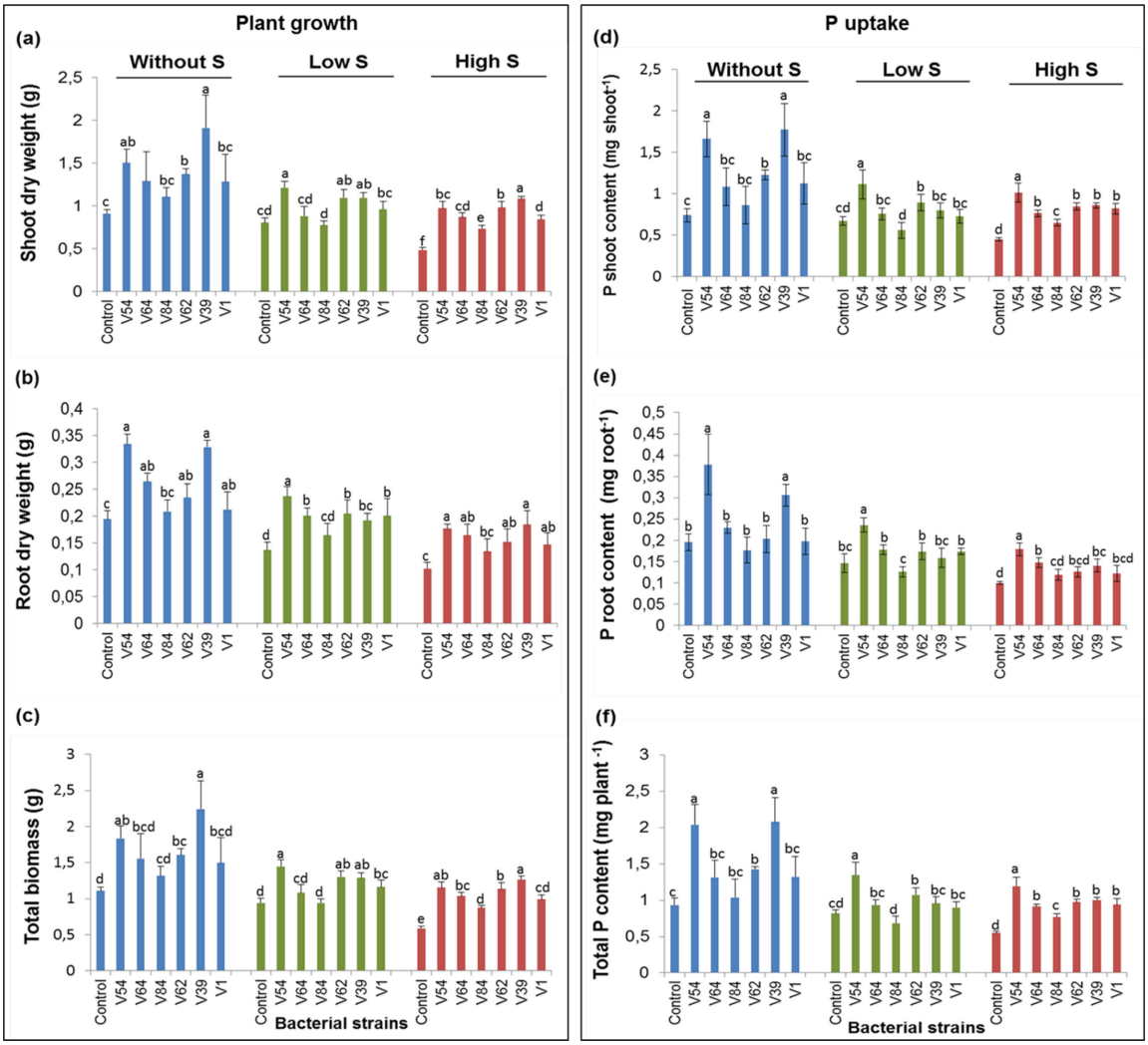


Figure 21. Bacterial inoculations promote tomato plant growth and P uptake under P and salt stress conditions. Effects of bacterial strains compared to the non-inoculated control on plant growth: (**a**) shoot, (**b**) root, (**c**) total biomass, and plant P uptake in (**d**) shoot, (**e**) root and (**f**) total P contents. Tomato plants grown under P stress and different levels of salt stress were assessed six weeks after planting in a greenhouse. Values are means of five replications and error bars are the standard deviation. The different letters indicate significant differences between treatments within a specific stress ($p < 0.05$) using the Tukey HSD test. All plants were under phosphorus stress, amended with hardly accessible Cameroonian RP (CRP). Without S = phosphorus stress without salt stress, Low S = phosphorus stress + low salt stress, and High S = phosphorus stress + high salt stress. Control (non-inoculated), V54, V64 and V84 (*Arthrobacter* sp.), V62, V39 and V1 (*Bacillus* sp.).

4.5. Discussion

In this study we selected bacterial isolates to test, primarily based on their phosphate-solubilizing activity, as well as their salinity tolerance. Notably, in agreement with previous report (Upadhyay et al., 2009), our results confirm that bacteria related to Bacillus genera reveal high salt tolerance. Most interestingly, the Bacillus strains V62 was able to grow at 10% NaCl, while the related strain V39 could develop even up to 14% NaCl. Although halotolerant bacteria can survive at high salt concentrations, they do not require such salt concentrations for their growth (Anton, 2014). They are able to withstand high salt concentrations due to their

capability to accumulate compatible osmolytes to maintain intracellular osmotic balance. Indeed these bacteria are crucial for agricultural production and may help plants survive under growth inhibitory levels of salt (Sharma et al., 2016). The use of halotolerant PGPR is an effective approach that has been employed successfully in various crops to improve their growth and tolerance under salt stress conditions (Sharma et al., 2016). Our findings confirm this potential.

Since the motility of bacteria is also an important factor to consider when selecting bacteria as bio-inoculants, it is interesting to note that all bacterial strains tested in this study showed swimming and swarming motility under normal conditions and some, mainly the *Bacillus* strains, even under high salt concentrations. This confirms previous reports indicating the motility of bacterial strains of different genera, including *Arthrobacter* and *Bacillus,* under normal and saline conditions (Becker et al., 2018; Singh and Jha, 2016; Vicario et al., 2015). Plant colonization is a complex process, and motility of bacteria in soil and/or on plant surfaces is a basic component of this process. Our findings suggest that the bacterial strains investigated here have the potential to effectively colonize the root of the host plant, even under saline conditions, which is the first and foremost step for plant-microbe interactions.

We used an easy to handle and fast solid plate method to check a high number of different rock phosphates or phosphate compounds with selected bacterial strains, since bacterial rock phosphate solubilizing activity is known to be strain specific and dependent on environmental conditions (Fankem et al., 2014; Tchuisseu Tchakounté et al., 2018). Strains able to solubilize a wider range of different phosphate compounds, as we demonstrate in our experiments, are likely to be efficient candidates to improve plant growth under P deficiency.

The major mechanism of mineral phosphate solubilization in plant-associated bacteria is the production of low molecular weight organic acids, which trigger acidification of the soil or media (Oteino et al., 2015; Rodriguez and Fraga, 1999). These organic acids can chelate the cation bound to phosphate with their hydroxyl and carboxyl groups (Kpomblekou-A and Tabatabai, 1994). The observed pH decrease in NBRIP medium supplied with TCP from an initial value of 7.0 to a final value of 4.9 indicates that acidification of medium due to organic acid production could facilitate bacterial phosphate solubilization. This is consistent with earlier reports showing that mineral phosphate solubilization is accompanied by a decrease in pH (Oteino et al., 2015). Surprisingly, in NBRIP medium supplied with CRP the decrease in pH was also observed in non-inoculated media (the control), suggesting that organic production is not the sole reason for the increase in P concentration in the culture medium, as reported earlier (Kim et al., 1997). Physical-chemical processes also contribute to the complex P transfer mechanisms in soils or soil water complexes. An alternative mechanism to organic acid production for solubilization of mineral phosphates is the release of hydrogen ions (H^+) to the

outer surface in exchange for cation uptake, or with the help of H^+ translocation ATPase (Alori et al., 2017; Rodriguez and Fraga, 1999). For example, assimilation of NH_4^+ together with H^+ excretion supports phosphate solubilization (Alori et al., 2017). It emerges from these results that phosphate-solubilizing mechanisms vary, depending on the nature of the phosphate source and the biological processes around. However, the additional application of bacterial strains significantly increased P uptake by the plants compared to application of Cameroonian RP alone.

Although all bacterial strains used in this study were able to solubilize phosphate under salt stress, it was observed that increasing salt concentrations could influence phosphate solubilization either negatively or positively depending on the bacterial strain and the phosphate source. Some bacteria, mainly *Bacillus* strain V39, showed higher phosphate solubilizing activity with increasing salt concentrations regardless of the phosphate source. Srinivasan and coworkers previously reported that *Aerococcus* and *Pseudomonas* spp. were able to solubilize TCP at different NaCl concentrations (Srinivasan et al., 2012). The significant increase in phosphate-solubilizing activity that we observed at 2% NaCl for all tested bacterial strains in the presence of different phosphate sources means that the selected bacterial strains actually require a moderate NaCl concentration for better solubilization. These findings are consistent with previous studies showing that phosphate solubilization by bacteria increased in the presence of salt (Cherif-Silini et al., 2013). Although some bacteria have been reported to show phosphate solubilizing activity at NaCl concentrations of up to 10% (Rosado et al., 2002), for most of our bacterial strains we observed that the efficiency of solubilizing the two phosphate sources significantly decreased at 4% NaCl. This decrease can be explained in two ways: (i) salt adversely affects bacterial growth and cell proliferation resulting in a loss of solubilization efficiency, or chloride ions (Cl^-) sequester or neutralize protons or acids produced in the media (Cherif-Silini et al., 2013).

In this study, the combined effects of abiotic stresses, i.e. P deficiency and salinity, significantly reduced tomato plant growth, although P stress had a more pronounced impact. This confirms earlier findings that under the combined effects of P and salt stresses, the most growth-limiting factor for barley is P deficiency (Talbi Zribi et al., 2011). Others (Shenker et al., 2003; Yousfi et al., 2007) reported similar results in corn and pea also grown under combined nutrient deficiency and salt stress conditions. We observed that the combined effects of P stress and salinity on plant growth and P uptake were most highlighted under high salt concentrations. Under low salt concentrations, the additive effect of P deficiency and salinity did not always affect plant growth and P uptake. Cultivated tomato is in any case a moderately tolerant plant, regulating water and ionic homeostasis (Martinez-Rodriguez et al., 2008), and is able to withstand certain concentrations of salt. Thus P deficiency seems to have a stronger impact on tomato plant growth, as confirmed in the present work.

Under P starvation, the effect of inoculating tomato plants with *Arthrobacter* and *Bacillus* strains significantly increased shoot and root dry weight by 50.4-109.6% and 9.3-72.1%, respectively, compared to the non-inoculated tomato plant under the same conditions. These values are higher than those of Zhang et al. who found that inoculation of tomato plants by *Acinetobacter* and *Ochracterum* increased shoot dry weight by 26.2-32.6% and root dry weight by 25.6-33.1% compared to non-inoculated plants under P starvation (Zhang et al., 2014). Our tomato plants inoculated with *Bacillus* strain V39 showed the highest biomass and P uptake increase of 103% and 122%, respectively, compared to the non-inoculated tomato plants supplied with Cameroonian RP without salt stress. This result is in accordance with a recent study showing that *Pantoea* sp. and *Bacillus* sp. contributed better to the plant growth of soybean inoculated with native bacteria isolated from Cameroonian palm tree rhizosphere, in pots supplied with Moroccan RP (Fankem et al., 2015). Bacterial inoculation with local RP is an economical and sustainable strategy for improving the growth and nutrient accumulation of plants in P-deficient, salt-affected soils. It is already established that bacterial inoculation efficiently increases the bioavailability of P in soils fertilized with RP (Fankem et al., 2014; Gomes et al., 2014). However, our present study has demonstrated for the first time that P availability for tomato plants improved under additional salt stress.

Under high salt stress our selected bacterial strains even exceeded the plant growth-promoting responses of the P-deficient negative control (without salt stress). The observed positive effects of *Arthrobacter* and *Bacillus* strains on tomato plant growth under combined P and salt stresses is comparable with results from a recent study reporting that bacterial strains, including *B. megaterium*, *Enterobacter* sp. *Providencia* sp. and *Ensifer adhaeren*, enhanced plant height and biomass of peanut plants supplied with TCP under salt stress (Jiang et al., 2018). Since the inoculated plants were not supplied with any additional source of soluble P, the higher amount of P detected in the shoot or roots of inoculated plants, as well as growth promotion under the different stress conditions may be attributed to the bacterially assisted phosphate solubilization effect from RP. The salt stress tolerance of the plants is perhaps increased by moving the equilibrium of P and other ions in the salt towards P. This clearly demonstrates that bacteria possessing phosphate solubilizing and salt tolerance abilities, as used in this study, play an important role in promoting plant growth under combined P and salt stresses.

Our findings also suggest that the higher the abiotic stress (P deficiency and salinity), the higher the bacterial effect on plant growth. In fact, some bacterial strains, especially *Arthrobacter* strain V54 and *Bacillus* strains V39, promoted higher growth under double stress conditions in relation to the respective stressed control, compared to P-deficiency treatment alone. This could be due to the fact that under increasing combined P and salt stresses, natural adaptation mechanisms of the plant are strongly reduced, and the bacterial effect on the plants becomes stronger. However, the difference between the effects of the single bacterial strains on

tomato plant growth, observed in this study, may be attributed to their individual traits and rhizosphere competencies.

It is important to select bacterial strains adapted to the environmental conditions where they are later expected to be applied. Our results show that the most efficient bacterial strains possessing various PGP activities, *Arthrobacter* strain V54 and *Bacillus* strainV39, also induced higher and more stable effects *in vivo* under the different applied stress conditions. Thus our study extends the understanding of PGP properties contributed by members of *Arthrobacter* and *Bacillus* genera, and provides important insights into the use of plant-microbe interactions to improve plant growth under combined phosphorus and salt stress conditions. Additionally, to the best of our knowledge, the present study is also the first to report the contribution of *Arthrobacter* sp. to the growth of tomato crops under combined P and salt stresses, and the first reporting the positive effect of PGPR on plant growth *in vivo* through solubilization of RP under saline conditions.

4.6. Conclusion

For the first time we demonstrate that *Arthrobacter* and *Bacillus* strains help tomato plants cope better with double stresses of salinity and P deficiency in a greenhouse experiment. Our study highlights the capacity of the selected bacteria to solubilize phosphate in the presence of high salt concentrations and to promote tomato plant growth under P deficiency and even under combined P and salt stresses. This provides valuable information for producing effective bio-fertilizers based on the combined application of rock phosphate and halotolerant phosphate-solubilizing bacteria, and offers promising potential to improve plant growth in P-deficient and salt-affected soils. However, since natural environments are more complex than experimental greenhouse conditions, field studies will be necessary to confirm such findings before these strains can be recommended as bio-fertilizers for commercial applications.

CHAPTER 5: RESPONSE OF FOUR MAIZE CULTIVARS TO *ARTHROBACTER* AND *BACILLUS* STRAINS INOCULATIONS UNDER COMBINED PHOSPHORUS AND SALT STRESS CONDITION

This chapter is based on the manuscript**:** G. V. Tchuisseu Tchakounté, B. Berger, S. Patz, M. Becker, V. Turečková, O. Novák, D. Tarkowská, H. Fankem, S. Ruppel (2020). The response of maize to inoculation with *Arthrobacter* sp. and *Bacillus* sp. in phosphorus-deficient, salinity-affected soil. *Microorganisms*, *8*, 1005

5.1. Abstract

Phosphorus (P) deficiency and salt stress are two major constraints of plant productivity. One strategy for reducing the effects of these stressors and for enhancing plant growth is to inoculate the plant with growth - promoting rhizobacteria (PGPR) under adverse soil conditions. Another strategy is the breeding and cultivation of stress-adapted plant cultivars. However, little is known how the plant cultivar and the growing conditions effect the growth promoting potential of PGPR. Four local maize cultivars (two composites (CMS 8704 and CMS 8501) and two hybrids (CHH 101 and CHH1013)) were, therefore, first investigated for their growth performance under P deficiency (plants supplied with hardly accessible Cameroonian rock phosphate) or sufficient P (plants supplied with soluble phosphate: KH_2PO_4) with or without addition of salt (EC = 12 ds m^{-1}). Then, they were evaluated for inoculation response with three *Arthrobacter* and three *Bacillus* strains under double P and salt stress condition. The bacterial strains, with different abilities to solubilize seven different inorganic phosphate sources, to fix atmospheric N_2 and to tolerate salt, based on previous studies were further *in vitro* tested for their phytohormone production. All tested strains showed the ability to produce indole acetic acid (IAA), abscisic acid (ABA), gibberellins (GAs) and cytokinins (CKs). Results from a first experiment demonstrated that nutrient uptake of all maize cultivars was significantly reduced under combined P and salt stress, and that growth of all maize cultivars was nearly equally negatively affected by the double stress. In a second experiment, it was shown that *Arthrobacter* and *Bacillus* inoculations improved Na^+ exclusion and K^+ uptake in maize plant and enhanced the plant growth under double P and salt stress condition compared to the non-inoculated control plants. Interestingly, the capacity of bacterial plant growth- stimulation (performed in a greenhouse) was higher in composite than in hybrid cultivars, although the growth performance of hybrid cultivars with or without bacterial treatment was still better compared to the composite cultivars. The results of our study imply that selected *Arthrobacter and Bacillus* strains improve plant growth in salt - affected and P - deficient soils to different degrees depending on maize cultivars. Hence, simultaneous to selecting good bacterial inoculants, it is advisable to select plant cultivars that benefit to a higher degree from association with these bacteria.

5.2. Introduction

Phosphorus (P) deficiency and salinity are two major constraints that decrease crop production around the world. In Cameroon, salty soils represent a considerable proportion of arable lands, covering about 671 thousand hectare (Kouam et al., 2017) and 85 % of soils are known to be acidic (Fankem et al., 2014). In acidic soils, P solubility is reduced due to the formation of sparingly soluble compounds with iron and aluminum. Moreover, P solubility is considerably decreased in salt-affected soils due to high Na^+ concentrations (Abbas et al., 2018). Maize (*Zea mays* L.) is an important cereal crop that is widely grown in various agro-ecological environments, and its growth is greatly reduced by high salinity and P deficiency (de Azevedo Neto et al., 2006). Maize growth and physiological responses either to salinity stress or to P deficiency have been extensively studied (Farooq et al., 2015; Hassan et al., 2018; Kholová et al., 2009; Liu et al., 2018; Ramos-Artuso et al., 2018), although the combination of both stressors has been neglected in many studies. A series of adaptive strategies have been reported to help maize plants cope either with excessive salinity or P deficiency. To overcome both the osmotic and ionic effects of salinity, salt-tolerant plants have better osmotic adjustment capabilities, enabling them to uptake water under high salinity levels and higher K^+/Na^+ ratio in the cytosol. Under P deficiency, plants increase root length, lateral root density as well as root hair number (Abbas et al., 2018). Studies on maize growth responses to combined effects of salinity and low P are still scarce.

In Cameroon, maize productivity is estimated at approximately 2.1 tons $hectare^{-1}$ as opposed to 11 tons $hectare^{-1}$attainable by developed countries (Takam-Fongang et al., 2019). Selection and breeding of composite and hybrid seeds have always been conducted to achieve high yield and better quality of crops under stressful conditions (Niu et al., 2017). While composite breeds are described as resistant to diseases and open pollinated, hybrid breeds are characterized by their high yield. Expansion of the cultivated area and adoption of improved cultivars resistant to salinity and acidity are some of the measures that have been used by farmers to manage soil acidity and salinity in Cameroon (Mapiemfu-Lamare et al., 2012). Nevertheless, these strategies are not effective since the country still remains a maize importer. It is well known that performance of plant cultivars not only depends on genetic traits, but also underlies continuing variable environmental conditions. A suitable cultivar is a crucial requirement for improvement of plant growth under a particular condition. Therefore, screening of maize cultivars for growth response to salt and P stress conditions appears to be a decisive criterion in selecting adapted cultivars for promoting their cultivation in salt-affected and P-deficient regions. To increase cultivar growth, it is not enough to just pursue only one strategy. In addition to breeding strategies, the success of cultivars can be enhanced by the use of effective

native plant growth-promoting rhizobacteria (PGPR) bearing phosphate solubilizing and salt tolerance abilities.

Application of indigenous PGPR in agriculture fits within the integrated soil fertility management (ISFM) concept and represent an affordable way for improving crop yields under different stress conditions in a sustainable way (Hussein and Joo, 2018). Based on the ISFM concept, it will be crucial to correctly match the appropriate PGPR with the respective/ suitable plant variety under certain environmental conditions to achieve the best results on plant growth and yield production (Pérez-Montaño et al., 2014). Besides phosphate solubilization and salt tolerance potential, the growth-promoting effect caused by the inoculation of PGPR results also from their capacity to produce phytohormones such as: indole acetic acid (IAA), abscisic acid (ABA) and gibberellins (GA), to fix atmospheric nitrogen and to increase the plant tolerance to pests as reported by Dartorta and co-workers (Dartora et al., 2016). Among PGPR, halotolerant phosphate solubilizing bacteria (PSB) have recently attracted the attention of agriculturists to improve plant growth and yield in P-deficient and salt-affected soils (Jiang et al., 2018). Although reports on enhancement of plant growth through these halotolerant and phosphate solubilizing bacteria, belonging to various genera, including *Bacillus* spp. and *Arthrobacter* spp. under salt stress and P deficiency as a separate growth-limiting factor are widely available, there had been paucity of information on the performance of bacteria on maize plants under combined salinity and P deficiency effects. In previous studies *Arthrobacter* and *Bacillus* strains were shown to increase peanut growth under combined P and salt stress by converting insoluble phosphate into soluble forms under saline condition and making it available to plants (Jiang et al., 2018). However, Long and colleagues reported that PGPR that induce growth in one plant species do not necessarily have similar effects on other species (Long et al., 2008). Even some bacteria show strong host-plant specificity and colonize only a single plant species or a limited variety of species (Batista et al., 2018; Long et al., 2008). Understanding how the plant growth-promoting properties of bacterial strains are affected by plant cultivar is widely recognized as a key to improving the level and reliability of plant growth stimulation by PGPR (Egamberdieva, 2010).

Previously, three *Arthrobacter* strains (V54, V64 and V84) and three *Bacillus* strains (V62, V39 and V1) were isolated from the rhizosphere of *Zea mays* in Cameroon and identified based on their partial 16S rRNA gene sequence (Tchuisseu Tchakounté et al., 2018). The six bacterial strains were selected and characterized. These strains stood out having several plant growth-promoting traits, such as ability to solubilize different rock phosphates, fix atmospheric nitrogen, tolerate salt, produce siderophores and finally improve maize germination rate and seedling growth (Tchuisseu et al. 2018). These bacterial strains were also shown to be capable of solubilizing phosphate under saline condition (Tchuisseu et al. 2019, Submitted). Thus, in

this study, we evaluated the response of four indigenous maize cultivars widely grown in Cameroon to the inoculation of previously selected *Arthrobacter* and *Bacillus* strains (Tchuisseu Tchakounté et al., 2018) under combined salt stress and P stress condition. We aimed to prove the plant cultivar dependent interaction between selected efficient native PGPR and maize composites and hybrid cultivars. Hence, we hypothesized that: (1) maize cultivars would respond differently to combined P deficiency and salinity; (2) bacterial inoculation would reduce the detrimental effect of combined P and salt stress on plants and the bacterial growth-promoting effect would be higher in hybrids than in composite cultivars. Accordingly, our main goals were: (i) to check the adverse effect of salt, P and combined P and salinity stress on maize plant growth; (ii) to assess the potential of bacterial strains to mitigate combined P and salt stress on maize plants; and (iii) to determine the phytohormones production ability of bacterial strains.

5.3. Material and methods

5.3.1. Plant material

Seeds of four maize (*Zea mays* L.) varieties, two composites (Cameroon maize series: CMS 8704 and CMS 8501) and two hybrids (Cameroon Highland Hybrids: CHH 101 and CHH103) were obtained from the Institute of Agricultural Research for Development (IRAD) and used as experimental materials. The cultivars characteristics are detailed in Table 11.

Table 11: Characteristics of the four different composites and hybrids maize cultivars used

Cultivar name	Cultivar type	Yield (t/ha)	Cycle	Germination rate	Year of release	Percentage of adoption	Grain properties
CMS 8704	Composite	4-6	110-120	94.5%	1987	9%	yellow, resistant to foliar disease and sweet taste, open pollinated
CMS 8501	Composite	4-6	110-120	90%	1985	8%	white, resistant to foliar disease and open pollinated
CHH 101	Hybrid	7-10	110-120	85%	1994	-	yellow and white, high yield
CHH 103	Hybrid	7-10	110-120	80%	1994	-	white yield and high yield

5.3.2. Plant growth

Seeds of four maize (*Zea mays* L.) cultivars CMS 8704, CMS 8501, CHH 101 and CHH 103 were used for a greenhouse experiment to assess their growth response to salt- and P stress conditions. Maize seeds for each cultivar were surface-sterilized in 4 % (v/v) sodium hypochlorite (H_2O_2) for 10 min, then washed five times with sterile distilled water and subjected to sterility checks on standard nutrient agar medium to ensure sterilization efficiency. The seeds were sown in quartz sand and maintained in a phyto-chamber (25/20 °C day/ night temperature) for 14 days for pre-germination. After germination, seedlings were transferred to plastic pots containing 1 L of mixed quartz sand and vermiculite (1/1), one plant per pot. Maize plants of the four cultivars were grown under the following four different growth conditions (treatments): 1. control - plants optimal fertilized (Hoagland solution with soluble phosphate + EC = 0 ds m^{-1}), 2. salt stress (optimal fertilization + EC = 12 ds m^{-1}), 3. P stress (RP + EC = 0 ds m^{-1}), 4. Combined P and salt stress (EC = 12 ds m^{-1} + RP).

The control treatment was fertilized with 30 mL Hoagland solution five times a week containing optimal easily plant available P concentration (KH_2PO_4, equivalent to P fertilization of 80 kg P ha^{-1}), and 30 mL osmose water supplied two times a week. P stress was triggered by fertilization with hardly accessible Cameroonian rock phosphate (RP). RP was washed 4 times with warm water in the following cycle: 1 h - 2 h – 1 h - 24 h, then dried at 60°C until complete evaporation of water and homogenized before use. All substrate in pots under P stress was mixed with Cameroonian rock phosphate at the rate of 350 mg P kg^{-1} soil. In the P stress treatment, potassium compensation was provided by adding a supplementary amount of K_2SO_4, in order to maintain the same K concentrations in all treatments. All other nutrients were applied as in the control treatment, but Hoagland solution lacking P was used. For the salt stress treatment, 20% NaCl was added to the Hoagland solution until reaching the targeted concentration (EC = 12 ds m^{-1}). In the combined stress treatment salt and P stress were triggered as in the single stress treatments described above, but 20% NaCl was added to Hoagland solution lacking in P. All treatments were 6 times replicated for each plant cultivar in a completely randomized block design. Plant growth was documented over six weeks after transplanting in a greenhouse. In all experiments, greenhouse conditions during plant cultivation were as follow: day/night temperature 25/23°C and 75% air humidity.

5.3.3. Plant Harvest and plant growth parameters measurement

At harvest, roots and shoots were separated. The root length and stem length were recorded. The fresh shoot and root weights were determined. Roots were washed with deionized water

and blotted with filter paper. Dry weights were recorded after oven-drying samples at 60°C for 72 h. P, K and Na in plant samples were determined as previously reported in chapter 2 (2.16).

5.3.4. Bacterial inoculation effect on maize growth under combined P and salt stress

All the four maize cultivars CMS 8704, CMS 8501, CHH 101 and CHH 103 were also used in a greenhouse experiment under Combined P and salt stress condition to evaluate the inoculation effect of the six bacterial strains: (*Arthrobacter* strains V54, V64 and V84; *Bacillus* strains V62, V39 and V1) on plant growth and nutrient uptake. Maize seeds for each cultivar were surface-sterilized as previously described and inoculated by immersion in 3 mL of respective bacterial suspension (microbial treatments) or 3 mL buffer solution (control treatments) for 15 min, then sown in quartz sand and germinated in a phyto-chamber (25/20°C day/ night temperature) for 14 days. Afterwards, seedlings were transferred in pots containing 1L of mixed quartz sand and vermiculite (1/1). All plants were grown under Combined P and salt stress condition. Salt and P stress was applied as described above. One day after transplanting the seedlings, they were re-inoculated with 2.5 mL of the respective bacterial suspension or with buffer solution for the control treatments. Watering and greenhouse growth conditions were the same as mentioned above. The experimental design was a completely randomized block system with seven treatments (six bacterial treatments and one control), four maize cultivars and six replications each. The harvest was carried out six weeks after transplanting and the growth parameters were determined as described above.

5.3.5. Phytohormones analysis

To measure the amount of phytohormones produced by bacteria, 1 mL of the bacterial suspensions adjusted to OD 0.2 at 620 nm was used to inoculate flasks containing 50 mL of standard nutrient broth culture medium supplemented with 1 g L^{-1} L-tryptophan. Glass vials containing the bacterial isolates were incubated for 48 h with continuous shaking at 28°C. After incubation, bacterial cells were harvested by centrifugation at 4°C for 10 min at 12000 rpm for the next steps.

Measurements of auxins (IAA) and cytokinins

Endogenous levels of cytokinins (CKs), free indole-3-acetic acid (IAA) and its catabolite 2-oxindole-3-acetic acid (oxIAA) were determined by LC-MS/MS methods (Novak et al., 2008; Novak et al., 2012). Approx. 5 mg of lyophilized bacterial cells were homogenized and extracted in 1 mL of modified Bieleski buffer (60% MeOH, 10% HCOOH and 30% H_2O). Alternatively, 1 mL of the liquid culture was diluted with 10 mL of modified Bieleski buffer. A mix of stable isotope-labeled internal standards (0.25 pmol of CK bases, ribosides, *N*-glucosides, 0.5 pmol of CK *O*-glucosides and nucleotides, 5 pmol of $^{13}C_6$-IAA and $^{13}C_6$-oxIAA per sample) was added to each sample to validate phytohormone determination (Novak et al.,

2008). The extracts were purified using two solid phase extraction columns, the octadecylsilica-based column (C18, 500 mg of sorbent, Applied Separations) and after that the Oasis MCX column (30 mg mL^{-1}, Waters) (Antoniadi et al., 2015). The analytes were eluted by three-step elution using a 60 % (v/v) MeOH, 0.35 M NH_4OH aqueous solution and 0.35 M NH_4OH in 60% (v/v) MeOH solution. Two alkaline eluates containing cytokinin metabolites were further purified by immunoaffinity extraction (Novák et al., 2003). The levels of CKs, free IAA, and oxIAA were determined using an UHPLC-ESI (-/+) -MS/MS system, in which an Acquity UPLC I-Class System device was coupled to a Xevo TQ-S MS device (Waters Corp.). Stable isotope-labelled internal standards were included for reference.

Abscisic acid (ABA) and Gibberellins (GA) production test

To determine the content of ABA using ultra performance liquid chromatographyelectrospray-mass spectrometry (UHPLC-ESI (-/+)-MS/MS), an aliquot of bacterial suspension containing 5 mg cells were shaken in an MM301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany) at 27 Hz for 3 min in the presence of 3 mm diameter tungsten carbide beads. An internal standard containing 20 pmoL (+) 3',5',5',7',7',7'-2H6-ABA (OlchemIm, Olomouc, Czech Republic) and 1 mL ice-cold methanol/water/acetic acid (10/89/1, v/v) were added to each of the samples. After a 1 h period of shaking in the dark at 4 ◦C, the preparations were centrifuged (20,000× *g*, 10 min, 4 ◦C) and the pellets re-extracted in a 0.5 mL extraction solvent for 30 min. The combined extracts were purified by passage through an OasisfiHLB cartridge (60 mg, 3 mL) (Waters Corp.), then evaporated to dryness in a Speed-Vac (UniEquip, Planegg, Germany), and finally analyzed by UHPLC-ESI (-/+)-MS/MS (Tureckova et al., 2009). The sample preparation and analysis of GAs was performed according to the method described in Urbanová et al. (Urbanová et al., 2013) with some modifications. Liquid bacterial media were prepared for ultra-trace analysis of GAs as follows: 5 mL of 100% acetonitrile was added to 5 mL of bacterial media (protein precipitation) with addition of 17 internal GAs standards ([2H2]GA1, [2H2]GA3, [2H2]GA4, [2H2]GA5, [2H2]GA6, [2H2]GA7, [2H2]GA8, [2H2]GA9, [2H2]GA15, [2H2]GA19, [2H2]GA20, [2H2]GA24, [2H2]GA29, [2H2]GA34, [2H2]GA44, [2H2]GA51, and [2H2]GA53; purchased from OlChemIm, Czech Republic) and incubated at -20 ◦C for 60 min. The samples were then centrifuged at 3180 rcf and 4 ◦C for 20 min. The supernatant was removed and evaporated to dryness in vacuo. The sample residue after evaporation was extracted with 1 mL of ice-cold 80% acetonitrile containing 5% formic acid and samples were further purified using reversed-phase and mixed mode SPE cartridges (Waters, Milford, MA, USA) and analyzed by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS; Micromass, Manchester, UK). GAs were detected using a multiple-reaction monitoring mode of the transition of the ion [M–H]- to the appropriate product ion. The Masslynx 4.1 software (Waters, Milford, MA, USA) was used to

analyze the data and the standard isotope dilution method was used to quantify the GAs levels (Rittenberg and Foster, 1940).

5.3.6. Statistical analyses

All data were analyzed statistically using the analysis of variance test. Mean comparison between treatments was conducted using the Tukey HSD test. Significance was determined at 5% ($p \leq 0.05$) probability level, and significantly different means were indicated by different letters. All the statistical calculations were performed using Sigma Plot software version 12.3.

5.4. Results

5.4.1. Hybrid maize cultivars show better performance than composite one

Maize cultivars significantly ($p < 0.05$; Tukey HSD test) differed in their growth behavior under the tested growth conditions, no stress, salt stress (= salinity), P stress (= phosphorus deficiency), and Combined P and salt stress (Fig.22; Fig.34 see appendices).

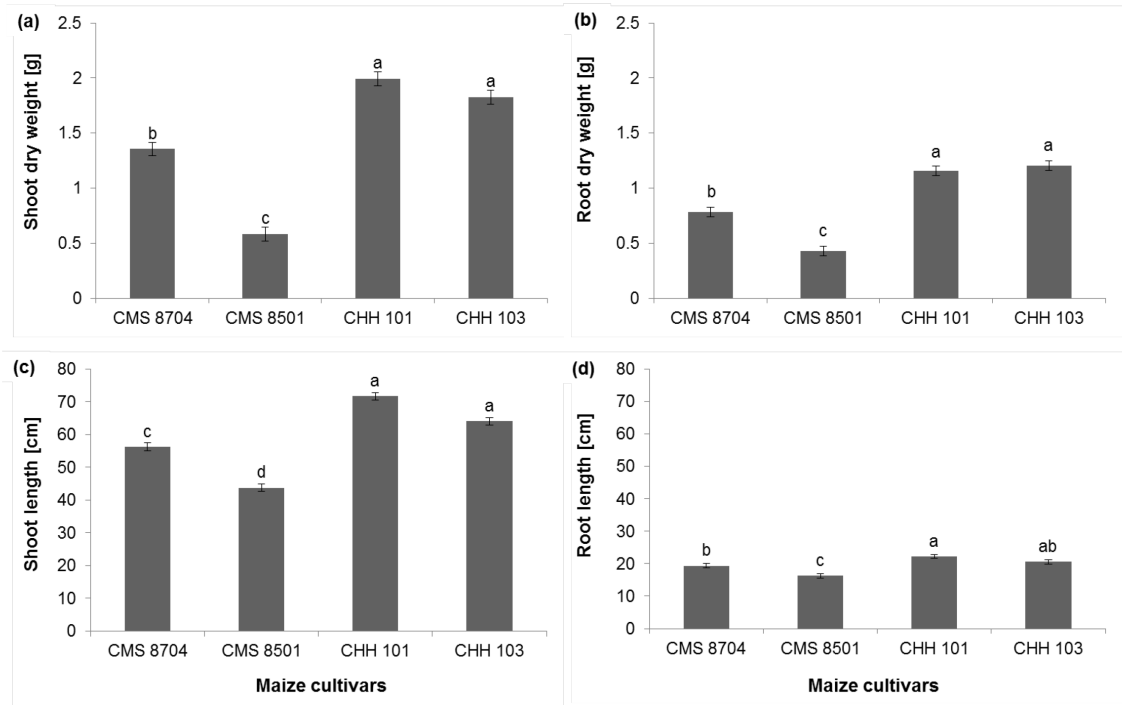


Figure 22. Mean of growth (averaged over all growth conditions) after 8 weeks under normal, salt stress, P stress, and Combined P and salt stress conditions: shoot dry weight (a), root dry weight (b), shoot length (c) and root length (d) of two composites (CMS 8704 and CMS 8501) and two hybrids (CHH101 and CHH 103) maize cultivars. Values are the means ± standard deviation of six replicates. For each cultivar, values sharing the same letter are not significantly different ($p \leq 0.05$) using the Tukey HSD test.

In general, hybrid cultivars (CHH 101 and CHH 103) showed a better shoot and root growth than the composites (CMS 8704 and CMS 8501), calculated as mean values over all four growth conditions (Fig.22). The two hybrid cultivars showed nearly the same growth respond, while composite ones reacted differentially. The composite CMS 8501 was the cultivar with the

lowest growth ability under our greenhouse conditions, followed by the CMS 8704 (Fig. 22). Shoot and root lengths data supported these results (Fig. 22).

5.4.2. Maize cultivar response specifically to salt, P and double P and salt stress conditions

First, we ensured that the proposed stress conditions induced plant growth reduction as intended (Fig. 23). The maize shoot and root dry weight decreased significantly under salt stress, followed by P stress. Moreover, the weight was dramatically reduced under combined P and salt stress. This trend was observed in shoot and dry weights and shoot length, except in root length which was not affected by salinity, combined P and salt stress, and even increased under P stress alone (Fig.23).

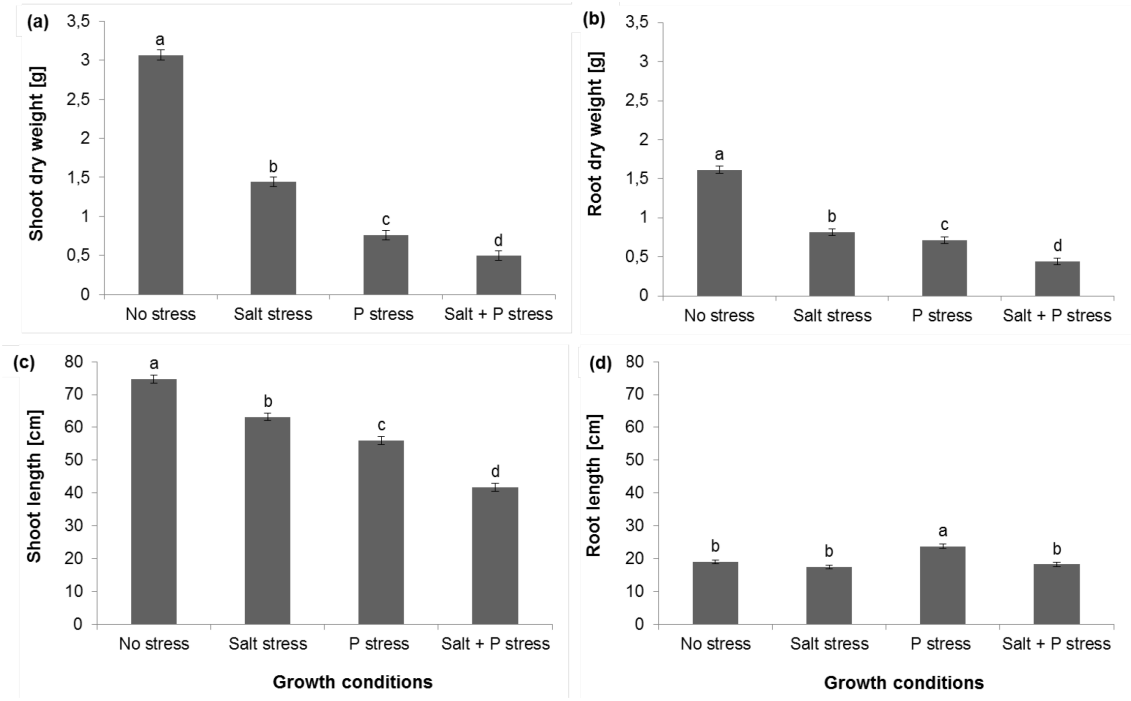


Figure 23. Salinity, phosphorus deficiency, and Combined P and salt stress reduced growth of different maize cultivars. Bars present growth mean (averaged over all maize cultivars) of salinity, P deficiency, and Combined P and salt stress on shoot dry weight (**a**), root dry weight (**b**), shoot length (**c**) and root length (**d**) of two composites (CMS 8704 and CMS 8501) and two hybrid maize cultivars (CHH101 and CHH 103). Values are means ± standard deviation of six replicates. For each cultivar, values sharing the same letter are not significantly different ($p \leq 0.05$) using the Tukey HSD test.

The four maize cultivars did not differ in their growth responses to salt stress. In salt treatment, nearly all four maize cultivars had the same reduction in shoot and root dry weights by about 50% (Fig.24). P stress effects on cultivars CMS 8704, CMS 8501 and CHH 103 were similar, varying from 77.6 -78.9% and 54.7- 60.5 % in shoot and root, respectively. Likewise, in combined P and salt stress maize cultivars CMS 8501, CHH 101 and CHH 103 showed the same growth reduction, only on a slightly higher level by about 80% and 70% in the shoot and root dry weights, respectively. The highest susceptibility to double salt + P stress was found in

composite cultivar CMS 8704 (Fig.24). It showed up to 92% and 82% reduction respectively in shoot and root dry matter production when compared to the non-stressed normally fertilized control plant. The cultivar which coped best with P stress was CHH 101 with only 69% and 48% reduction in shoot and root dry weights, respectively (Fig.24).

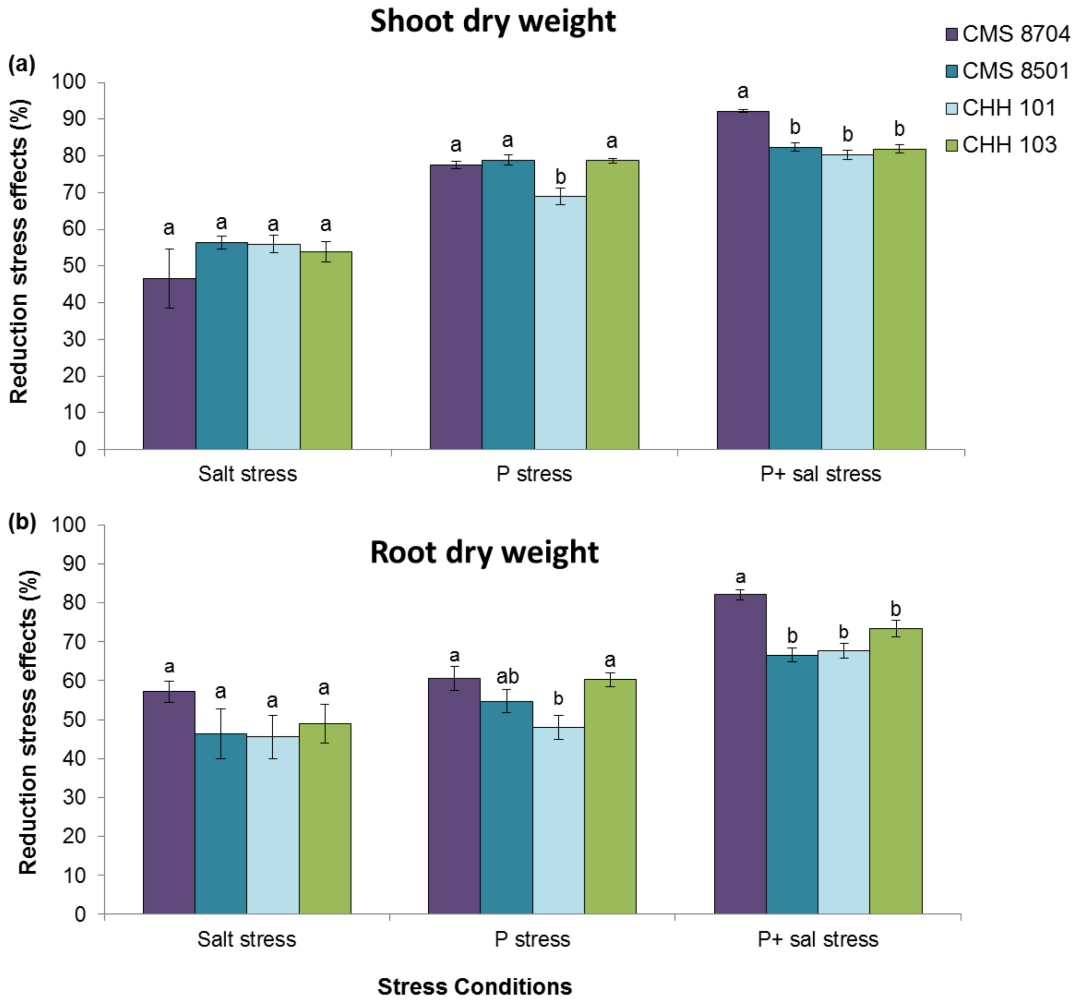


Figure 24. Salinity, phosphorus deficiency, and Combined P and salt stress effect on growth of different maize cultivars (two composites (CMS 8704 and CMS 8501) and two hybrids (CHH101 and CHH 103)) regarding shoot dry weight (**a**) and root dry weight (**b**). Values are the means ± standard deviation of six replicates. For each cultivar, values sharing the same letter are not significantly different ($p \leq 0.05$) using the Tukey HSD test.

5.4.3 P deficiency, salinity, and double P and salt stress affect nutrient concentrations of maize plants

The analysis of variance showed significant effects ($p \leq 0.001$) of growth conditions, varieties and their interaction on K^+, Na^+, and P concentrations in maize organs. Results of the effects of salinity, P deficiency and combined P and salt stress on nutrient concentrations of the four maize cultivars are presented in Table 12. Shoot and root Na^+ concentrations increased significantly ($p < 0.05$; Tukey HSD test) in all four maize cultivars in the salinity treatments

with increasing Na^+ concentrations in combined P and salt stress compared to the control plants grown under normal fertilization conditions. P stress alone did not affect the shoot and root Na^+ concentration in either cultivar (Table 12).

Shoot and root K^+ concentrations decreased significantly ($p < 0.05$; Tukey HSD test) in all four maize cultivars in response to all stress conditions, with a most pronounced effect of combined salt + P stress. Salt stress reduced the shoot and root K^+ concentrations more than the P stress in all cultivars. Shoot and root K^+/ Na^+ ratios decreased significantly ($p < 0.05$; Tukey HSD test) in all maize cultivars under salt and P stress (Table 12), with the lowest ratio under salt stress. The K^+/ Na^+ ratios in all cultivars in the combined salt + P stress treatment declined to similar values as in the salinity treatment.

Shoot and root P concentrations significantly declined ($p < 0.05$; Tukey HSD test) in all cultivars in the P stress and combined salt + P stress treatments, while salt stress alone showed no effect on shoot and root P concentration of all maize cultivars (Table 12). Effects of combined salt + P stress on shoot and root P concentration was similar to that of P deficiency alone in all cultivars.

Table 12: Effects of salinity, P deficiency, and double P and salt stress on shoot and root K^+, Na^+ an P concentrations, and K^+/Na^+ ratios of the four maize cultivars.

Maize Cultivars	Growth conditions	Shoot				Root			
		(mg g^{-1})			K^+/Na^+ ratio	(mg g^{-1})			K^+/Na^+ ratio
		Na^+	K^+	P		Na^+	K^+	P	
CMS 8704	No stress	0.6 ± 0.3 ^{c}	79.2 ± 2.0 ^{a}	8.5 ± 1.5 ^{a}	150.7 ± 61.2 ^{a}	7.5 ± 4.2 ^{c}	56.9 ± 3.2 ^{a}	4.5 ± 0.7 ^{a}	9.2 ± 4.2 ^{a}
	salt	10.0 ± 0.8 ^{b}	65.1 ± 2.4 ^{b}	8.8 ± 0.7 ^{a}	6.5 ± 1.3 ^{c}	35.9 ± 4.2 ^{a}	27.0 ± 2.1 ^{b}	5.5 ± 1.2 ^{a}	0.8 ± 0.1 ^{b}
	P stress	1.3 ± 0.1 ^{c}	72.3 ± 2.2 ^{ab}	0.8 ± 0.02 ^{b}	55.1 ± 6.7 ^{b}	8.5 ± 1.9 ^{c}	42.5 ± 2.7 ^{c}	0.9 ± 0.1 ^{b}	5.2 ± 1.2 ^{ab}
	P +salt stress	35.0 ± 1.0 ^{a}	33.3 ± 1.4 ^{c}	0.7 ± 0.1 ^{b}	1.0 ± 0.03 ^{c}	26.9 ± 3.1 ^{b}	13.6 ± 1.5 ^{d}	0.7 ± 0.03 ^{b}	0.5 ±0.02 ^{b}
CMS 8501	No stress	0.5 ± 0.07 ^{c}	75.0 ± 2.8 ^{a}	8.2 ± 2.0 ^{a}	133.5 ± 13.1 ^{a}	6.8 ± 0.1 ^{b}	53.3 ± 5.0 ^{a}	4.6 ± 0.9 ^{a}	7.7 ± 0.8 ^{a}
	salt	11.5 ± 2.5 ^{b}	59.3 ± 2.5 ^{b}	8.7 ± 0.6 ^{a}	5.3 ± 1.0 ^{c}	26.1 ± 3.0 ^{a}	19.5 ± 0.5 ^{c}	3.7 ± 1.0 ^{a}	0.7 ± 0.09 ^{c}
	P stress	2.9 ± 1.9 ^{c}	71.4 ± 3.2 ^{a}	1.4 ± 0.4 ^{b}	34.2 ± 14.1 ^{b}	7.6 ± 1.5 ^{b}	34.2 ± 3.1 ^{b}	1.3 ± 0.1 ^{b}	4.6 ± 1.3 ^{b}
	P +salt stress	36.6 ± 1.9 ^{a}	49.4 ± 1.7 ^{c}	0.8 ± 0.01 ^{b}	1.3 ± 0.06 ^{c}	27.7 ± 0.7 ^{a}	16.2 ± 1.2 ^{c}	0.9 ± 0.08 ^{b}	0.6 ± 0.05 ^{c}
CHH 101	No stress	0.7 ± 0.2 ^{c}	81.1 ± 1.7 ^{a}	7.2 ± 0.9 ^{a}	125.0 ± 48.6 ^{a}	5.9 ± 0.4 ^{c}	51.2 ± 2.3 ^{a}	3.3 ± 0.3 ^{a}	8.7 ± 0.6 ^{a}
	salt	7.2 ± 1.0 ^{b}	59.9 ± 4.5 ^{b}	6.5 ± 0.3 ^{a}	8.5 ± 1.6 ^{c}	26.7 ± 3.3 ^{b}	20.9 ± 1.4 ^{c}	2.9 ± 0.3 ^{a}	0.8 ± 0.08 ^{c}
	P stress	1.6 ± 0.3 ^{c}	64.3 ± 1.5 ^{b}	1.3 ± 0.1 ^{b}	40.6 ± 9.4 ^{b}	9.4 ± 3.0 ^{c}	36.1 ± 2.5 ^{b}	1.0 ± 0.1 ^{b}	4.3 ± 1.7 ^{b}
	P +salt stress	28.1 ± 5.2 ^{a}	42.23 ± 2.2 ^{c}	0.8 ± 0.01 ^{b}	1.5 ± 0.2 ^{c}	31.2 ± 5.0 ^{a}	19 ± 1.4 ^{c}	0.7 ± 0.05 ^{b}	0.6 ± 0.08 ^{c}
CHH 103	No stress	0.6 ± 0.1 ^{c}	75.0 ± 3.6 ^{a}	7.2 ± 0.9 ^{a}	123.0 ± 17.7 ^{a}	5.9 ± 1.1 ^{c}	48.6 ± 2.1 ^{a}	3.5 ± 0.6 ^{a}	8.6 ± 2.3 ^{a}
	salt	10.8 ± 3.2 ^{b}	65.2 ± 2.0 ^{b}	8.5 ± 1.5 ^{a}	6.4 ± 1.5 ^{c}	28.4 ± 5.5 ^{b}	26.9 ± 2.0 ^{c}	4.5 ± 0.6 ^{a}	0.9 ± 0.2 ^{c}
	P stress	1.0 ± 0.3 ^{c}	66.7 ± 2.0 ^{b}	0.8 ± 0.04 ^{b}	71.8 ± 21.4 ^{b}	7.1 ± 0.8 ^{c}	40.4 ± 4.6 ^{b}	0.9 ± 0.07 ^{b}	5.7 ± 1.0 ^{b}
	P +salt stress	24.9 ± 3.0 ^{a}	35.3 ± 1.2 ^{c}	0.6 ± 0.03 ^{b}	1.4 ± 0.1 ^{c}	37.9 ± 1.8 ^{a}	16.7 ± 2.2 ^{d}	0.7 ± 0.06 ^{b}	0.4 ± 0.07 ^{c}

Maize plants grown under no stress (normal), salt stress, P stress, and double P and salt stress conditions were documented six weeks after planting in a greenhouse. P stress was applied using the hardly available Cameroonian rock phosphate and the salt stress on plants was induced by applying an electrical conductivity of 12 ds m^{-1}. Values are the means ± standard deviation of six replicates. For each cultivar, values sharing the same letter are not significantly different ($p \leq 0.05$) using the Tukey HSD test.

5.4.4. Bacterial inoculation improved plant growth and nutrient uptake of maize plant under combined P and salt stress

To alleviate the negative effects of combined salt + P stress, in the present study we tested the effect of six previously selected bacterial PGP strains from *Arthrobacter* and *Bacillus* genera. All selected PGP bacterial strains significantly ($p < 0.05$; Tukey HSD test) enhanced shoot and root dry weights (Fig.25) as well as shoot and root lengths (Fig. 35 see appendices) of maize plants which were grown under combined salt + P stress conditions compared to the respective non-inoculated control regardless of the maize cultivar.

In the composite cultivar CMS 8704 *Bacillus* strain V62 inoculation induced the highest shoot dry weight (0.99 g) and root dry weight (0.79 g) production (Fig.25 a, e). For *Bacillus* strains V39 (0.47 g) and V1 (0.42 g) respectively the greatest shoot and root dry weights was recorded in CMS 8501 (Fig. 25b, f). In CHH 101, the maximum shoot and root dry weight were respectively found in plant inoculated with *Bacillus* strains V62 (1,40 g; Fig. 25c) and V39 (1.27 g; Fig.25g), while plants of CHH 103 exhibited the highest shoot (1.13 g; Fig. 25d) and root (1.04g; Fig.25h) dry weights when inoculated with *Arthrobacter* V64.

Likewise, total plant biomass of all maize cultivars was significantly increased ($p < 0.05$; Tukey HSD test) by all bacterial strains, as compared to non-inoculated control plants (Fig. 26). However in most cases, plant inoculated with *Arthrobacter* V84 followed by *Bacillus* V1 had a lowest total plant biomass as compared to other inoculated plants. These are the strains compiling the lowest number of PGP traits compared to the other four herein tested strains (Tchuisseu Tchakounté et al., 2018)

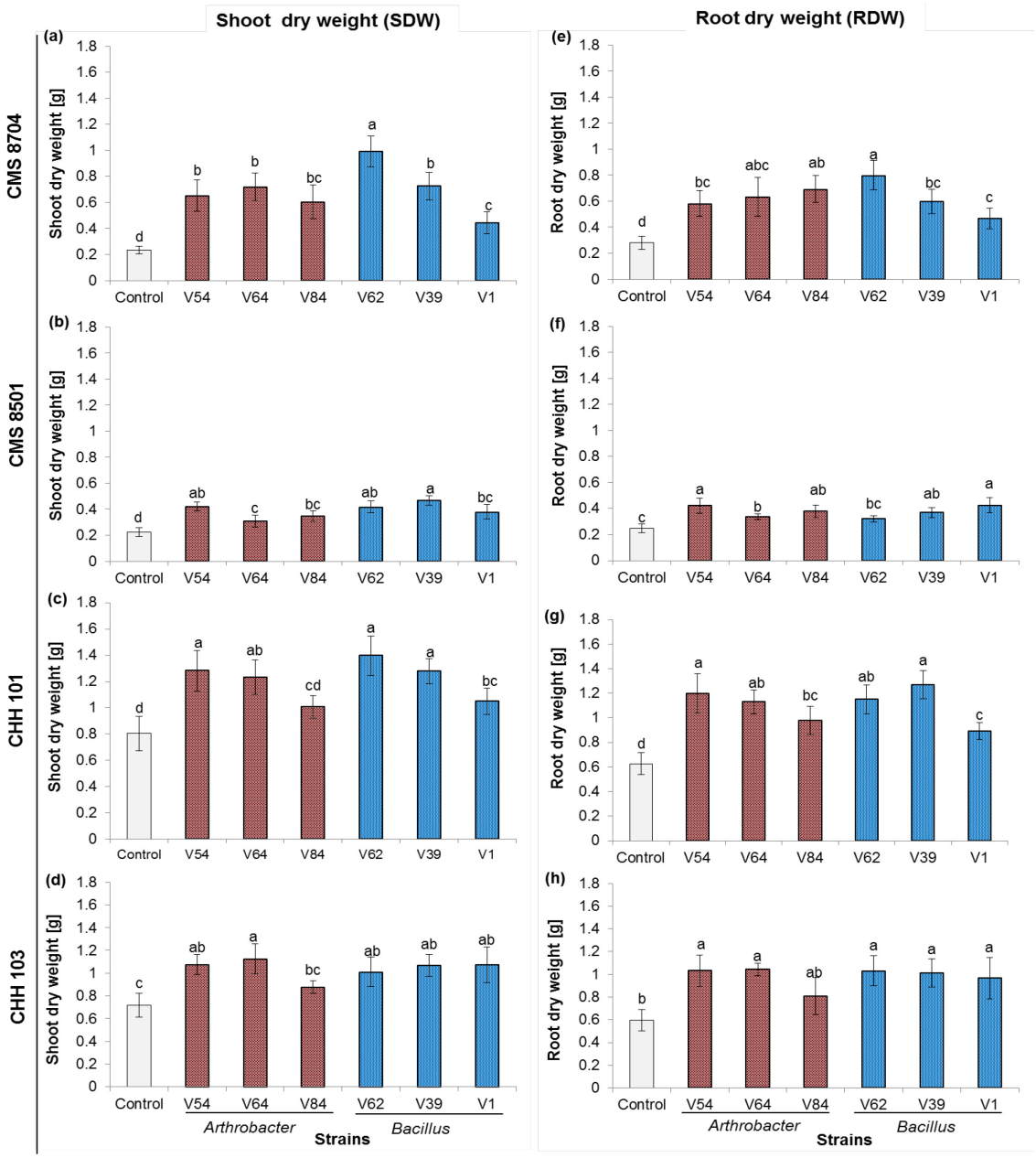


Figure 25. Bacterial inoculations promote shoot and root dry weights of different cultivars growing under Combined P and salt stress: shoot (**a-d**) and root (**e-h**) dry weights growth of different maize plants inoculated with *Arthrobacter* (V54, V64 and V84) and *Bacillus* (V62, V39 and V1) strains compared to the non-inoculated controls. Maize plants grown under Combined P and salt stress were assessed six weeks after planting in a greenhouse. Values are means of six replications and error bars represent standard deviations. Bars with same letter are not significantly different according to Tukey HSD test at ($p < 0.05$) within the same cultivar. P stress was applied using the hardly available Cameroonian rock phosphate and the salt stress on plants was induced by applying an electrical conductivity of 12 ds m^{-1}.

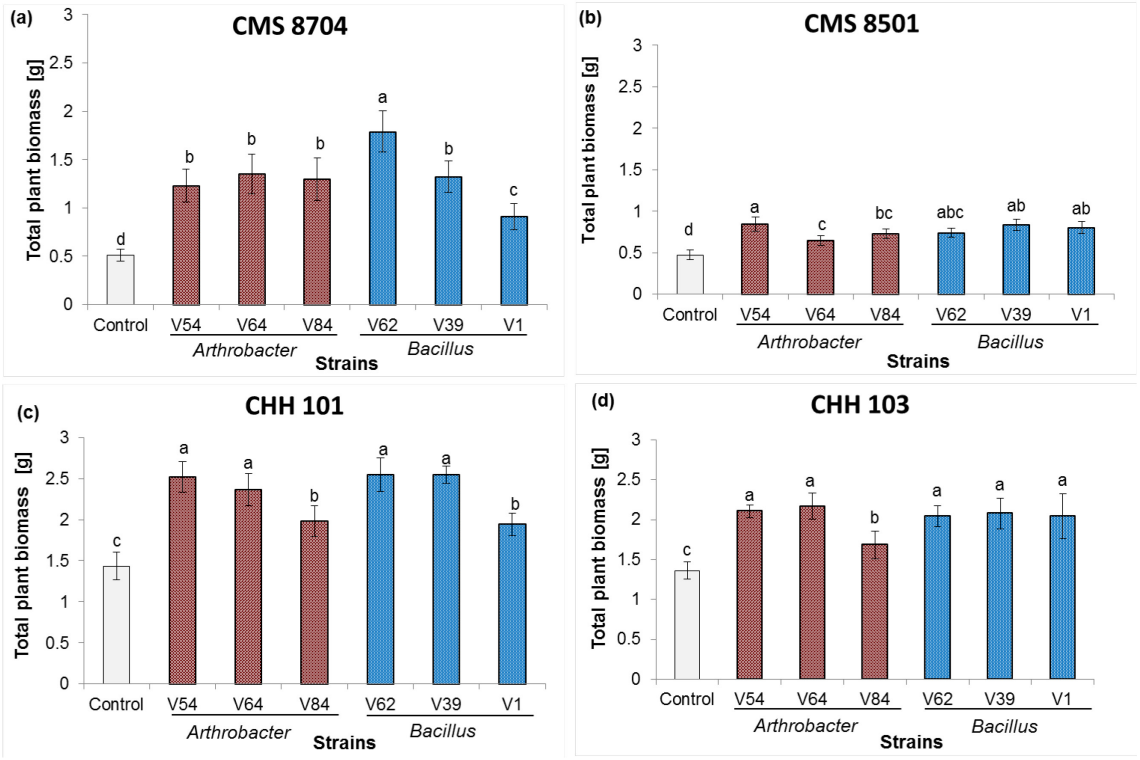


Figure 26. Bacterial inoculations enhanced total biomass of different maize cultivars growing under combined P and salt stress: total biomass of maize plants (**a**) CMS 8704, (**b**) CMS 8509, (**c**) CHH 101 and (**d**) CHH 103 inoculated with *Arthrobacter* (V54, V64 and V84) and *Bacillus* (V62, V39 and V1) strains compared to the non-inoculated control plants (Control). Maize plants grown under combined P and salt stress were assessed six weeks after planting in a greenhouse. Values are means of six replications and error bars represent standard deviations. Bars with same letter are not significantly different according to Tukey HSD test at ($p < 0.05$) within the same cultivar. P stress was applied using the hardly available Cameroonian rock phosphate and the salt stress on plants was induced by applying an electrical conductivity of 12 ds m^{-1}.

Although all bacterial strains showed a positive effect on all maize plants growth, when they were grown under double P and salt stress, there was a significant variation on bacterial inoculation effects between cultivars and between bacterial strains within the same cultivar. Bacterial inoculation effects on most parameters were higher in composite cultivars, mainly CMS 8704 than in hybrid cultivars CHH 101 and CHH 103 (Fig. 27). The same result was observed when cultivar means (averaged over all bacterial strains effect) were compared (Fig. 28). Composite CMS 8704, which was the most by the double P + salt stress affected cultivar, responded with the highest and tremendous shoot and root dry weight production due to PGPR application (Fig.27). There were also significant difference between the effect means (average over all maize cultivars) of the different strains on maize plant growth and *Arthrobacter* strains (V54 and V64) and *Bacillus* strains (V39 and V62) showed higher growth promoting - effects as compared to *Arthrobacter* V84 and *Bacillus* strain V1in most cases (Fig. 29).

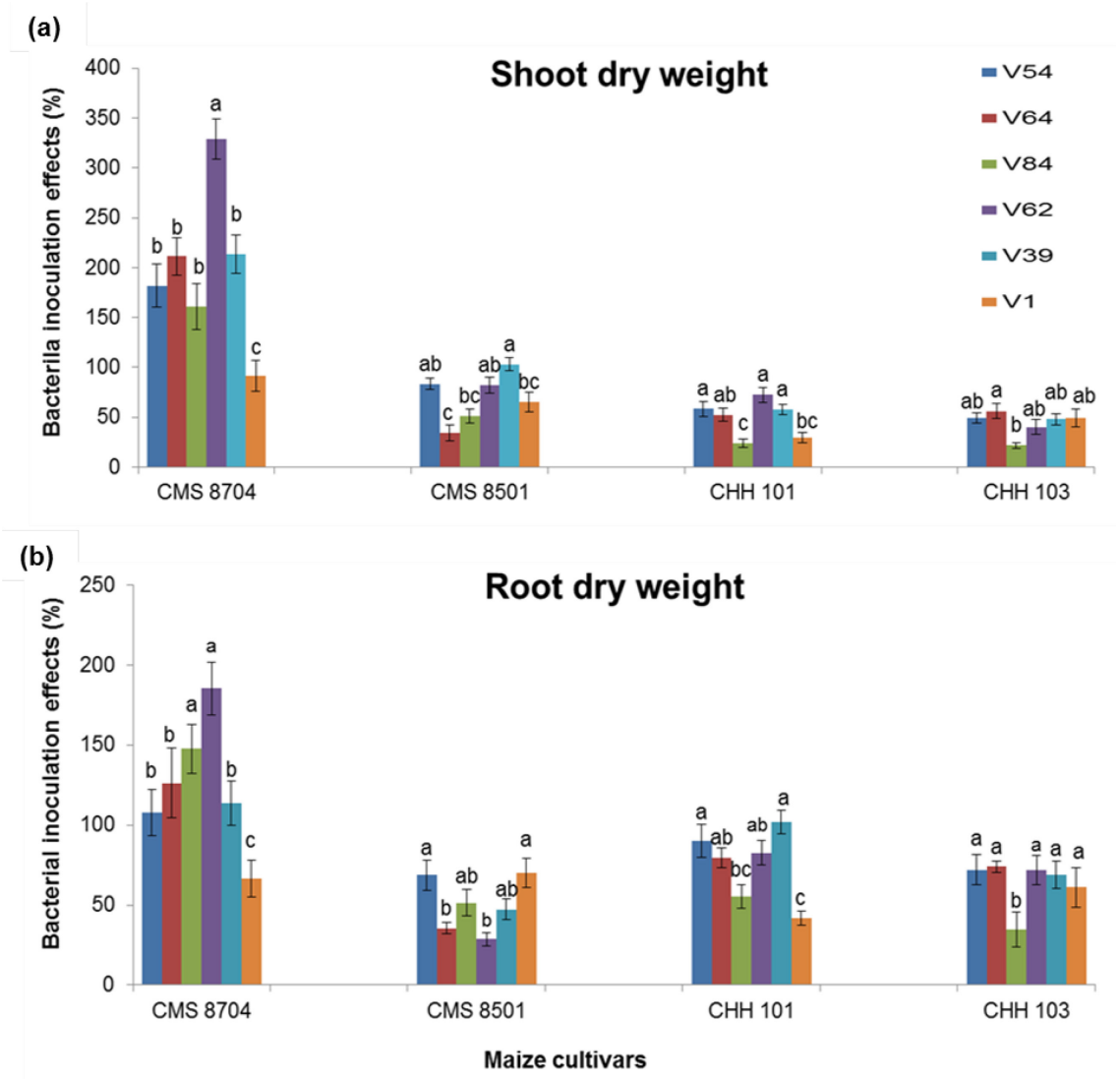


Figure 27. Plant growth-promoting effects of bacterial inoculants on maize cultivars under combined P and salt stress. Effects (%) of *Arthrobacter* and *Bacillus* strains compared to the non-inoculated control shoot dry weight (**a**) and root dry weight (**b**) of two composite (CMS 8704 and CMS 8501) and two hybrid (CHH 101 and CHH 103) maize cultivars. Maize plants grown under combined salt + P stress were assessed six weeks after planting in a greenhouse. Values are means of six replications and error bars represent standard deviations. Bars with same letter are not significantly different according to Tukey HSD test at ($p < 0.05$) within the same cultivar. P stress was applied using the hardly available Cameroonian rock phosphate and the salt stress on plants was induced by applying an electrical conductivity of 12 ds m^{-1}. Control (non-inoculated), V54, V64 and V84 (*Arthrobacter* spp.), V62, V39 and V1 (*Bacillus* spp.).

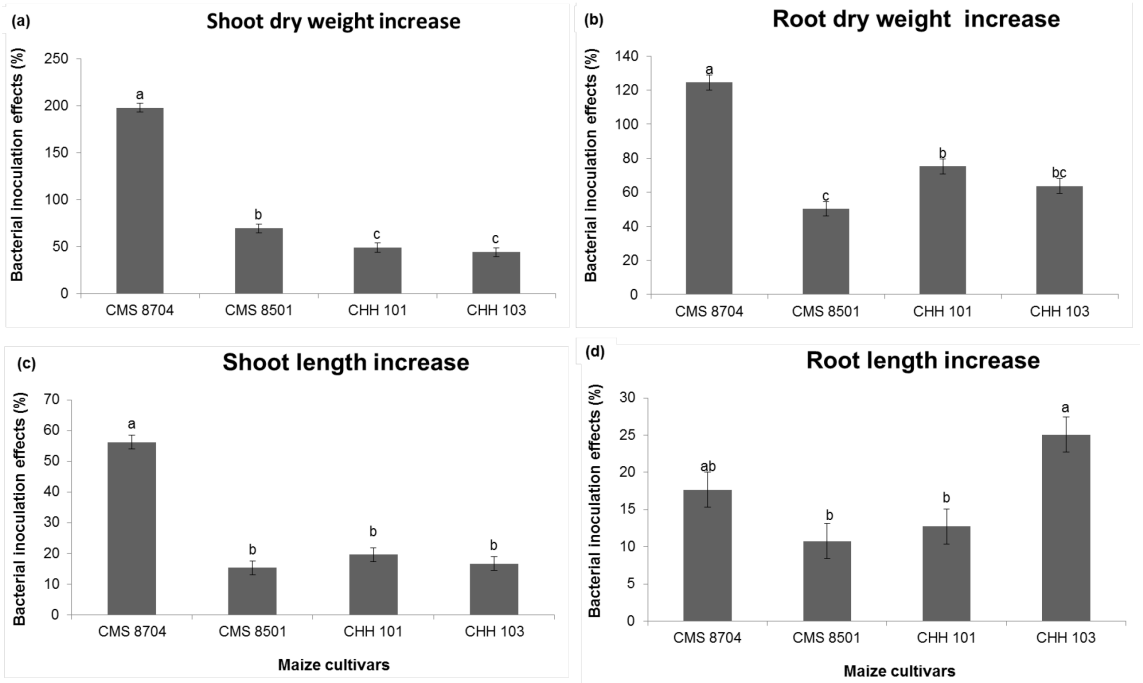


Figure 28. Differential cultivar response to bacterial inoculation effects: Mean increase in (a) shoot dry weight, (b) root dry weight, (c) shoot length and (d) root length (averaged over all bacterial inoculations with V54, V64, V84 (*Arthrobacter* sp.) and V62, V39, V1 (*Bacillus* sp.) of two composites (CMS 8704 and CMS 8501) and two hybrids (CHH101 and CHH 103) maize cultivars. Maize plants grown under Combined P and salt stress were assessed six weeks after planting in a greenhouse. Values are means of six replications and error bars represent standard deviations. Bars with same letter are not significantly different according to Tukey HSD test at ($p < 0.05$) within the same cultivar. P stress was applied using the hardly available Cameroonian rock phosphate and the salt stress on plants was induced by applying an electrical conductivity of 12 ds m^{-1}.

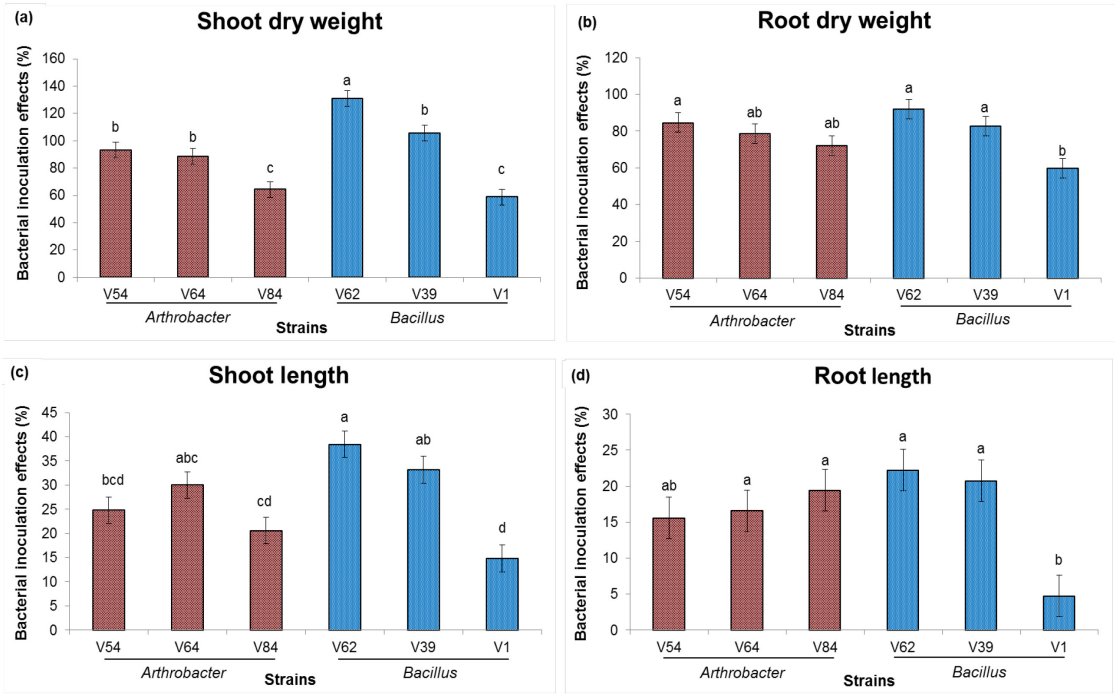


Figure 29. Mean effects (%) (averaged over all cultivars) of *Arthrobacter* (V54, V64 and V84) and *Bacillus* (V62, V39 and V1) strains compared to the non-inoculated control on shoot length (**a**), root

length (**b**), shoot dry weight (**c**), and root dry weight (**d**) of two composite (CMS 8704 and CMS 85 01) and two hybrid (CHH 101 and CHH 103) maize cultivars. Maize plants grown under combined P and salt were assessed six weeks after planting in a greenhouse. Values are means of six replications and error bars represent standard deviations. Bars with same letter are not significantly different according to Tukey HSD test at ($p < 0.05$) within the same cultivar.

5.4.5. Arthrobacter and Bacillus strains improved nutrient concentration of different maize cultivars under double P and salt stress

Combined P and salt stress increased Na^+ in plants. Bacterial inoculations decreased Na^+ concentrations in maize plant organs in all cultivars (Table 13; Fig. 30). Nevertheless, bacterial effect differed with the cultivar and the plant organ. All bacterial strains caused a decrease of shoot Na^+ concentrations in all maize cultivars, while this effect was not so clearly detected in the roots (Table 13). K^+ concentrations and K^+/Na^+ ratios in shoots and roots of all maize cultivars, when compared to their respective not inoculated control plants, were increased by bacterial inoculations (Table 13; Fig.30), demonstrating a clear beneficial bacterial impact on plant salt tolerance. Bacterial effect on K^+ concentrations and K^+/Na^+ ratios varied with the cultivar and the bacterial strain. If the K^+ concentration dropped down to 35 or 33 mg K g^{-1} shoot (Cultivars CHH103 and CMS 8704, respectively) due to induced combined P + salt stress, the plant cultivars responded to PGP application with an increased K^+ concentration and K^+/Na^+ ratio in the shoot dry matter (Table 13).

Table 13: Effects of inoculation with *Arthrobacter* and *Bacillus* strains on shoot and root K^+, Na^+ an P concentrations, and K^+/Na^+ ratios of the four maize cultivars

Maize Cultivars	Bacterial treatments	Shoot				Root			
		(mg g^{-1})			K^+/Na^+ ratio	(mg g^{-1})			K^+/Na^+ ratio
		K^+	Na^+	P		K^+	Na^+	P	
CMS 87 04	Control	33.3 ± 1.4 ^{b}	35.0 ± 1.1 ^{a}	0.6 ± 0.1 ^{b}	0.9 ± 0.03 ^{c}	13.6 ± 1.5 ^{c}	26.9 ± 3.1 ^{a}	0.7 ± 0.03 ^{b}	0.5 ± 0.02 ^{b}
	V54	53.6 ± 2.2 ^{a}	23.4 ± 1.3 ^{b}	0.8 ± 0.01 ^{a}	2.2 ± 0.1 ^{ab}	21.9 ± 1.2 ^{ab}	25.6 ± 2.7 ^{ab}	0.9 ± 0.1 ^{ab}	0.8 ± 0.09 ^{ab}
	V64	49.8 ± 4.3 ^{a}	22.0 ± 2.9 ^{b}	0.8 ± 0.08 ^{a}	2.3 ± 0.5 ^{ab}	24.4 ± 3.8 ^{a}	25.0 ± 1.0 ^{ab}	1.0 ± 0.09 ^{a}	0.9 ± 0.1 ^{a}
	V84	50.8 ± 5.5 ^{a}	23.5 ± 1.8 ^{b}	0.8 ± 0.03 ^{a}	2.1 ± 0.2 ^{ab}	23.7 ± 2.6 ^{a}	27.0 ± 6.2 ^{a}	0.8 ± 0.05 ^{ab}	0.9 ± 0.3 ^{a}
	V62	57.2 ± 3.9 ^{a}	21.7± 2.6 ^{b}	0.9 ± 0.02 ^{a}	2.6 ± 0.2 ^{a}	22.9 ± 2.2 ^{a}	24.7 ± 2.1 ^{ab}	1.0 ± 0.05 ^{a}	0.9 ± 0.02 ^{a}
	V39	52.1 ± 4.5 ^{a}	26.5 ± 2.9 ^{b}	0.8 ± 0.07 ^{ab}	1.9 ± 0.2 ^{ab}	17.2 ± 2.4 ^{bc}	28.5 ± 5.3 ^{a}	1.0 ± 0.1 ^{a}	0.6 ± 0.1 ^{b}
	V1	51.1 ± 3.2 ^{a}	28.2 ± 5.3 ^{ab}	0.9 ± 0.04 ^{a}	1.8 ± 0.4 ^{ab}	15.7 ± 1.7 ^{c}	18.2 ± 2.1 ^{b}	1.0 ± 0.06 ^{a}	0.8 ± 0.3 ^{ab}
CMS 8501	Control	49.4 ± 1.7 ^{ab}	36.6 ± 1.9 ^{a}	0.8 ± 0.01 ^{a}	1.3 ± 0.06 ^{c}	16.2 ± 1.2 ^{b}	27.7 ± 0.7 ^{a}	0.9 ± 0.08 ^{a}	0.5 ± 0.05 ^{c}
	V54	55.7 ± 1.5 ^{ab}	21.1 ± 2.9 ^{bc}	0.8 ± 0.07 ^{a}	2.6 ± 0.4 ^{a}	31.7 ± 5.3 ^{a}	24.9 ± 2.3 ^{ab}	1.1 ± 0.1 ^{a}	1.2 ± 0.09 ^{a}
	V64	59.6 ± 3.6 ^{a}	24.4 ± 2.1 ^{bc}	0.9 ± 0.05 ^{a}	2.4 ± 0.17 ^{ab}	23.8 ± 4.4 ^{ab}	23.2 ± 0.9 ^{b}	1 ± 0.05 ^{a}	1.0 ± 0.1 ^{b}
	V84	60.9 ± 0.7 ^{a}	22.7 ± 0.3 ^{bc}	0.7 ± 0.08 ^{a}	2.6 ± 0.06 ^{a}	16.4 ± 1.4 ^{b}	21.4 ± 3.5 ^{b}	1.1 ± 0.09 ^{a}	0.7 ± 0.06 ^{c}
	V62	56.7 ± 2.5 ^{ab}	19.3 ± 2.2 ^{c}	0.9 ± 0.06 ^{a}	2.9 ± 0.2 ^{a}	16.2 ± 1.8 ^{b}	27.4 ± 1.2 ^{a}	0.9 ± 0.01 ^{a}	0.5 ± 0.04 ^{c}
	V39	49.7 ± 7.2 ^{ab}	26.5 ± 2.0 ^{b}	0.8 ± 0.06 ^{a}	1.8 ± 0.3 ^{bc}	18.5 ± 0.8 ^{b}	23.6 ± 1.1 ^{ab}	0.9 ± 0.1 ^{a}	0.7 ± 0.001 ^{c}
	V1	45.8 ± 7.4 ^{b}	24.8 ± 2.5 ^{bc}	0.9 ± 0.04 ^{a}	1.8 ± 0.1 ^{bc}	18.2 ± 1.1 ^{b}	25.7 ± 3.0 ^{ab}	1.0 ± 0.07 ^{a}	0.7 ± 0.04 ^{c}
CHH 101	Control	42.2 ± 2.2 ^{a}	28.1 ± 5.2 ^{a}	0.8 ± 0.01 ^{b}	1.5 ± 0.2 ^{a}	19 ± 1.4 ^{a}	31.2 ± 5.0 ^{a}	0.7 ± 0.05 ^{ab}	0.6 ± 0.08 ^{a}
	V54	46.1 ± 2.6 ^{a}	19.0 ± 1.2 ^{b}	0.79 ± 0.05 ^{b}	2.4 ± 0.2 ^{a}	22.7 ± 2.6 ^{a}	31.8 ± 4.0 ^{a}	0.7 ± 0.09 ^{ab}	0.7 ± 0.1 ^{a}
	V64	49.4 ± 4.8 ^{a}	18.4 ± 6.3 ^{b}	0.7 ± 0.04 ^{b}	2.9 ± 1.2 ^{a}	21.1 ± 2.2 ^{a}	26.7 ± 2.0 ^{a}	0.6 ± 0.04 ^{b}	0.7 ± 0.07 ^{a}
	V84	47.1 ± 1.1 ^{a}	20.1 ± 3.3 ^{ab}	0.8 ± 0.05 ^{b}	2.3 ± 0.4 ^{a}	22.7 ± 1.0 ^{a}	29.0 ± 3.6 ^{a}	0.8 ± 0.08 ^{ab}	0.7 ± 0.1 ^{a}
	V62	50.0 ± 3.7 ^{a}	18.8 ± 2.5 ^{b}	0.7 ± 0.05 ^{b}	2.7 ± 0.5 ^{a}	22.4 ± 2.0 ^{a}	29.4 ± 2 ^{a}	0.7 ± 0.05 ^{ab}	0.7 ± 0.1 ^{a}
	V39	48.7 ± 4.5 ^{a}	18.3 ± 1.7 ^{b}	0.7 ± 0.03 ^{b}	2.6 ± 0.4 ^{a}	22.1 ± 1.0 ^{a}	28.6 ± 1.8 ^{a}	0.8 ± 0.05 ^{ab}	0.7 ± 0.08 ^{a}
	V1	49.8 ± 3.3 ^{a}	21.9 ± 3.1 ^{ab}	1.1 ± 0.1 ^{a}	2.3 ± 0.3 ^{a}	21.6 ± 1.3 ^{a}	28.7 ± 3.6 ^{a}	0.8 ± 0.03 ^{a}	0.7 ± 0.1 ^{a}
CHH 103	Control	35.2 ± 1.2 ^{c}	24.9 ± 3.0 ^{a}	0.6 ± 0.03 ^{d}	1.4 ± 0.1 ^{c}	16.7 ± 2.2 ^{c}	37.9 ± 1.8 ^{ab}	0.7 ± 0.06 ^{c}	0.4 ± 0.07 ^{b}
	V54	44.8 ± 4.1 ^{b}	20.3 ± 1.0 ^{ab}	0.7 ± 0.03 ^{c}	2.2 ± 0.2 ^{bc}	23.3 ± 3.1 ^{ab}	28.0 ± 5.5 ^{b}	0.8 ± 0.03 ^{abc}	0.8 ± 0.3 ^{a}
	V64	44.8 ± 3.8 ^{b}	15.4 ± 3.1 ^{bc}	0.9 ± 0.03 ^{a}	2.9 ± 0.3 ^{ab}	20.0 ± 1.2 ^{bc}	30.6 ± 5.3 ^{ab}	1.0 ± 0.1 ^{a}	0.6 ± 0.1 ^{ab}
	V84	47.1 ± 3.7 ^{ab}	13.6 ± 0.9 ^{c}	0.8 ± 0.008 ^{b}	3.4 ± 0.4 ^{a}	21.3 ± 2.6 ^{abc}	29.8 ± 4.4 ^{ab}	0.9 ± 0.08 ^{ab}	0.7 ± 0.1 ^{ab}
	V62	44.6 ± 2.8 ^{b}	13.6 ± 2.8 ^{c}	0.7 ± 0.009 ^{cd}	3.4 ± 0.9 ^{a}	22.2 ± 2.6 ^{abc}	30.9 ± 2.6 ^{ab}	0.7 ± 0.01 ^{bc}	0.72 ± 0.1 ^{ab}
	V39	44.4 ± 3.6 ^{b}	14.0 ± 1.9 ^{c}	0.9 ± 0.07 ^{a}	3.2 ± 0.6 ^{ab}	26.6 ± 2.6 ^{a}	27.9 ± 1.6 ^{b}	0.9 ± 0.07 ^{ab}	0.9 ± 0.09 ^{a}
	V1	53.1 ± 1.5 ^{a}	17.8 ± 1.3 ^{bc}	0.7 ± 0.02 ^{cd}	2.9 ± 0.2 ^{ab}	23.8 ± 3.5 ^{ab}	23.7 ± 2.7 ^{a}	0.8 ± 0.05 ^{abc}	1.0 ± 0.2 ^{a}

Maize plants grown under combined P + salt stress were assessed six weeks after planting in a greenhouse. Values are means of six replications and error bars represent standard deviations. Means with same letter are not significantly different according to Tukey HSD test at ($p < 0.05$) within the same cultivar. P stress was applied using the hardly available Cameroonian rock phosphate and the salt stress on plants was induced by applying an electrical conductivity of 12 ds m^{-1}. Control (non-inoculated), V54, V64 and V84 (*Arthrobacter* sp.), V62, V39 and V1 (*Bacillus* sp).

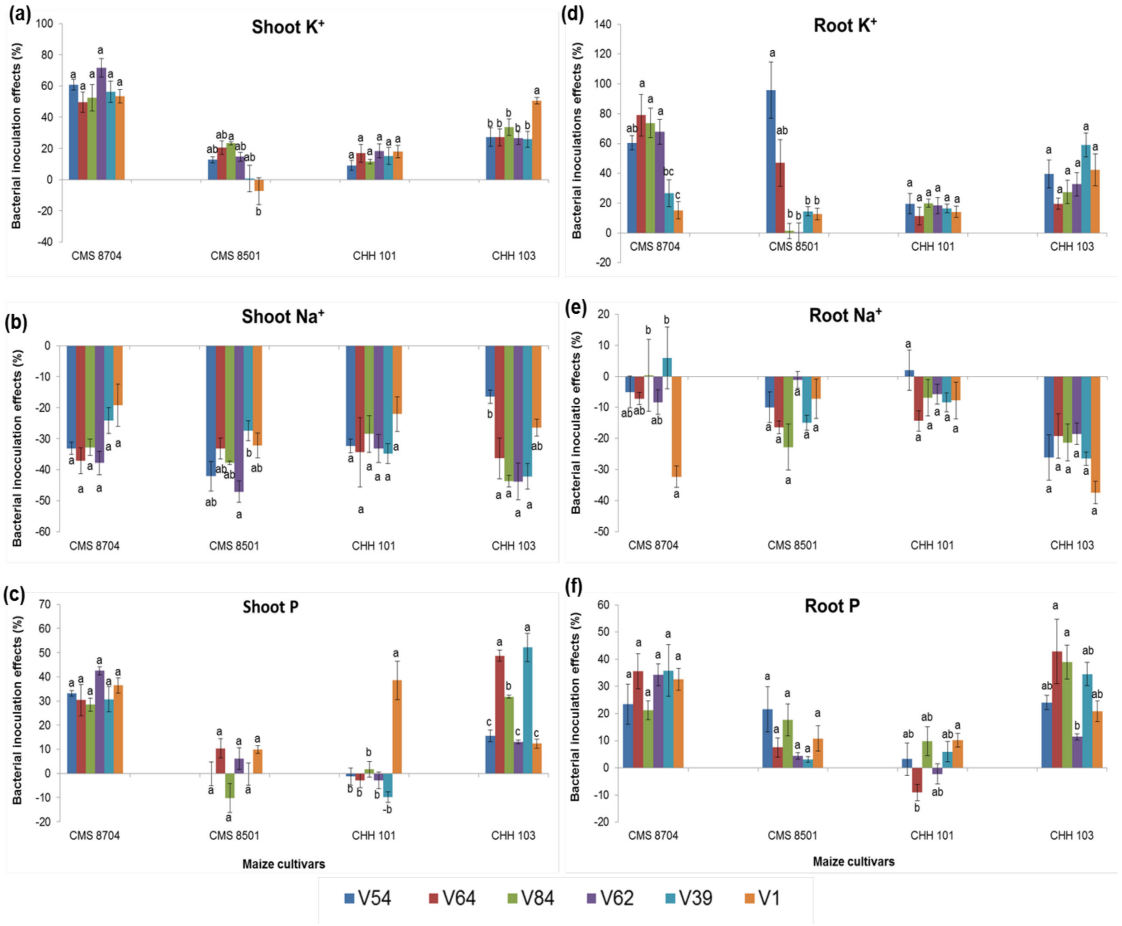


Figure 30. Bacterial inoculations showed differential effects on Na^+, K^+ and P concentrations of different maize cultivars under combined P and salt stress. Effects (%) of *Arthrobacter* (V54, V64 and V84) and *Bacillus* (V62, V39 and V1) strains compared to the non-inoculated controls on (**a**) K^+ shoot concentration, (**b**) Na^+ shoot concentration, (**c**) P shoot concentration, (**d**) K^+ root concentration, **(e)** Na^+ root content and **(f)** P root content of two composite (CMS 8704 and CMS 85 01) and two hybrid (CHH 101 and CHH 103) maize cultivars. Maize plants grown under combined P and salt stress were assessed six weeks after planting in a greenhouse. Values are means of six replications and error bars represent standard deviations. Bars with same letter are not significantly different according to Tukey HSD test at ($p < 0.05$) within the same cultivar. P stress was applied using the hardly available Cameroonian rock phosphate and the salt stress on plants was induced by applying an electrical conductivity of 12 ds m^{-1}.

Besides improvement of plant growth, *Arthrobacter* and *Bacillus* strains exhibited influences on P contents in plant organs. Significant beneficial effects of bacterial strains on total P uptake when compared to non-inoculated control plants were observed in plant inoculated with five strains (V39, V54, V62, V64 and V84), two strains (V39 and V54) and (V39 and V64) in CMS

8704, and CHH 101 and CHH 103, while bacterial effects on total P uptake in CMS 8501 were non-significant (Fig.31).

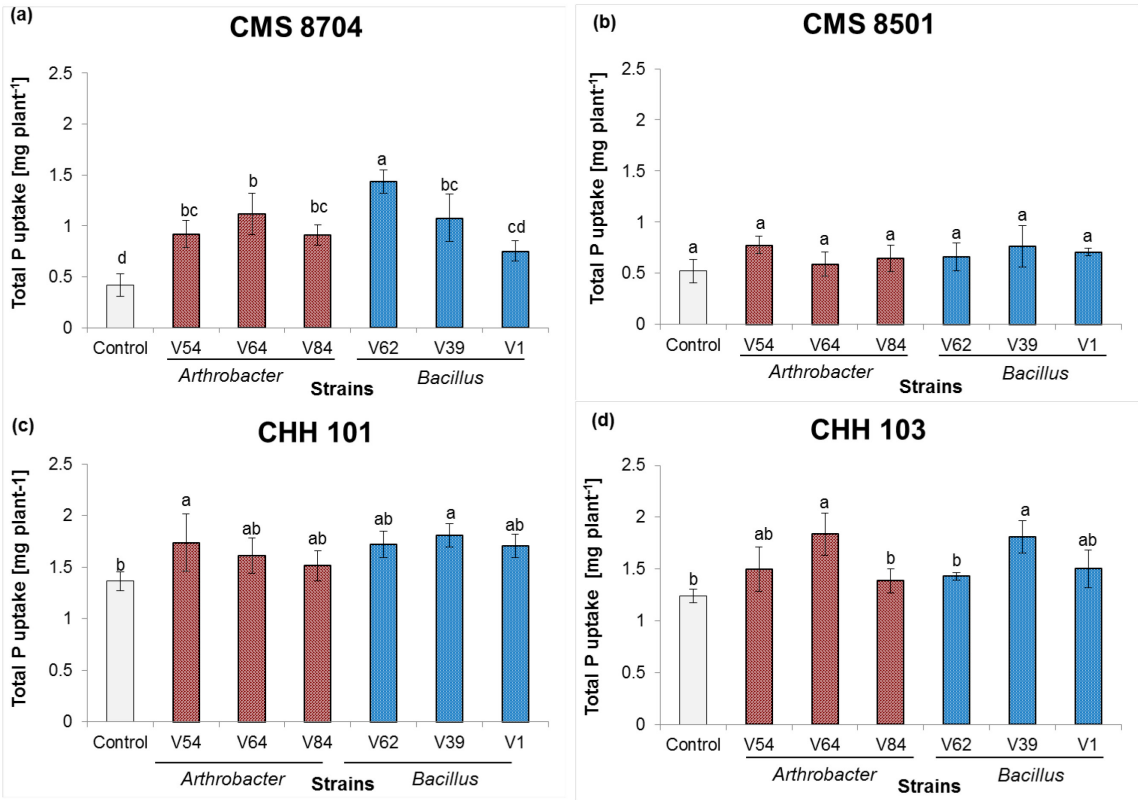


Figure 31. Total P uptake of different cultivars growing under Combined P and salt stress after bacterial inoculation. Effects of bacterial strains compared to the non-inoculated control on total P uptake of (**a**) CMS 8704, (**b**) CMS 8501, (**c**) CHH 101 and (**d**) CHH 103. Maize plants grown under combined salt + P stress were assessed six weeks after planting in a greenhouse. Values are means of six replications and error bars represent standard deviations. Bars with same letter are not significantly different according to Tukey HSD test at ($p < 0.05$) within the same cultivar. P stress was applied using the hardly available Cameroonian rock phosphate and the salt stress on plants was induced by applying an electrical conductivity of 12 ds m^{-1}. Control (non-inoculated), V54, V64 and V84 (*Arthrobacter* spp.), V62, V39 and V1 (*Bacillus* spp.).

5.4.6. Arthrobacter and Bacillus strains produced different types of phytohormones

All the six bacterial strains were assessed for their ability to produce plant growth promoting substances such as indole acetic acid (IAA), cytokinins, abscisic acid (ABA) and gibbellins (GA). Data presented in Table 14 reveal that all bacteria produced IAA in the presence of tryptophan. However, there was a significant variation in the IAA producing ability of the strains, ranging from 243- 6702.2 ng mL^{-1}. *Arthrobacter* strains were more efficient in producing IAA than *Bacillus* strains. The highest production was recorded for *Arthrobacter* V84, while the lowest was detected in *Bacillus* strain V39.

All bacterial strains were able to produce cytokinins and abscisic acid. Gibberelic acid such as GA1, GA3, GA4, GA5, GA7, GA8, GA9, GA13, GA15, GA19, GA20, GA24, GA29, GA34, GA44, GA51 and GA53 were detected in liquid medium inoculated with the different bacterial

strains (Fig. 32). Results showed significant differences ($p < 0.05$) in the capability of bacteria to produce the different GA compounds. Out of 17 GA detected, only three: GA1, GA8 and GA24 (highlighted in red color) were produced by all the six bacterial strains. Some GAs such GA5 and GA7 (highlighted in yellow) were only produced by *Bacillus* strains (Fig. 32). GA1, GA3, GA4 and GA7 were reported as the biologically active in bacteria (Shi et al., 2017). Therefore, we focused on these four compounds for further differences between GA producing abilities of strains. The highest GA1 (9.1ng mL^{-1}) and GA7 (17.5 ng mL^{-1}) was recorded in *Bacillus* strain V62, while *Bacillus* strain V1 and *Arthrobacter* strain V64 showed the *highest* efficiency in producing GA3 and GA4, respectively (Table 14).

Table 14: Phytohormones production efficiency of different isolates: efficacy of different *Arthrobacter* and *Bacillus* strains to produce indole acetic acid (IAA), cytokinins (Cks), abscisic acid (ABA) and gibberellins

		Phytohormone production (ng mL^{-1})						
Bacterial isolates		**Abscissic acid (ABA)**	**Auxin (IAA)**	**Cytokinins (CKs)**	**Gibberellins (GA)**			
					GA1	**GA3**	**GA4**	**GA7**
***Arthrobacter* strains**	V54	1.1 ± 0.2 ^{a}	4781.1 ± 397.2 ^{b}	38.3 ± 5.2 ^{ab}	3.7± 0.2^{b}	Nd	0.22 ± 0.04^{b}	0.01 ± 0.003 ^{b}
	V64	1.9 ± 0.3 ^{a}	5169.8 ± 223.7 ^{b}	27.1 ± 2.7 ^{c}	0.1±0.02 ^{d}	Nd	4.9 ± 0.4 ^{a}	Nd
	V84	1.3 ± 0.3 ^{a}	6702.2 ± 253.0 ^{a}	23.3 ± 1.1 ^{c}	2.0± 0.2^{c}	0.01 ± 0.002 ^{b}	Nd	Nd
***Bacillus* strains**	V62	2.0 ± 0.1 ^{a}	385.6 ± 22.6 ^{c}	29.2 ± 2.1 ^{bc}	9.1 ± 0.1 ^{a}	0.03 ± 0.01 ^{b}	4.2 ± 1.1 ^{a}	17.5 ± 0.6 ^{a}
	V39	1.2 ± 0.6 ^{a}	242.9 ± 50.1 ^{c}	42.6 ± 6.2 ^{a}	3.8 ± 0.7 ^{b}	0.07 ± 0.04 ^{b}	0.3 ± 0.02 ^{b}	0.1 ± 0.008 ^{b}
	V1	1.4 ± 0.1 ^{a}	279.8 ± 15.3 ^{c}	47.7 ± 1.3 ^{a}	0.9 ±0.1^{d}	0.16 ± 0.04 ^{a}	0.6 ± 0.08 ^{a}	0.17 ± 0.08 ^{b}

Nd: no detected, data are means ± S.D. of three replicates for each treatment. Different letters indicate differences among isolates ($p < 0.05$) using the Tukey HSD test.

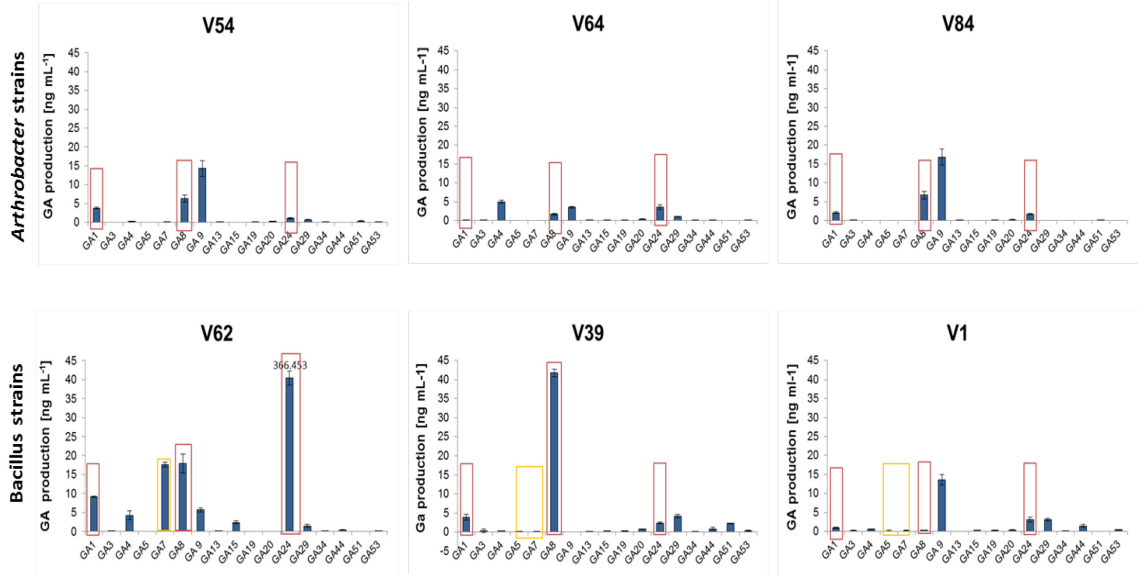


Figure 32. Gibberelins (GAs) production activity of different bacterial strains. Efficiency of *Arthrobacter* and *Bacillus* strains to produce the different GA compounds. GAs highlighted in red are produced by all bacterial and those in yellow are produced only by *Bacillus* strains.

5.5. Discussion

In the present study, four maize varieties, two composites (CMS 88704 and CMS 8501) and two hybrids (CHH 101 and CHH 103) were tested for their growth performance. Our results showed differential cultivar growth responses under adequate fertilization conditions, with hybrid cultivars exhibiting a better growth than the composite cultivars. In line with this statement, Hossain reported a better growth of hybrid maize cultivars compared to composites, when testing eight different maize varieties' responses (four hybrids and four composites) to Zn fertilization (Hossain et al., 2011). Abayomi et al (2007) linked the far better growth gain in hybrid cultivars to larger leaf growth and leaf surface area compared the open pollinated varieties (OPV, composite). Hybrid cultivars have been characterized to have high yield potentials mainly due to larger assimilatory surfaces and higher leaf angles that could facilitate diffusion of light into the lower portion of the canopy (Aderibigbe et al., 2017). However, yield potential and quality traits of cultivars vary under different environmental conditions.

Regarding salinity, P deficiency and the interactive effects of salinity + P deficiency maize cultivars responded differently. All maize cultivars were negatively affected by the applied salt stress in the same range to about 50% of shoot and root growth reduction. Cultivar specific differences were detected when P stress was induced by applying hardly available rock phosphate to the plant. Here the hybrid CHH 101 was the most efficient cultivar to cope with this P-deficiency. Nevertheless, when considering individual applied stress, we noted that P deficiency had a more negative impact on maize plants growth compared to salt stress. This result agreed with previous findings, demonstrating that, as compared to salinity, P deficiency was more detrimental to the growth of barley (Talbi Zribi et al., 2011), wheat (Abbas et al., 2018), and maize (Tang et al., 2019). We also observed that the effects of P deficiency and salt stress were additive, since under combined P and salt stress condition, the growth reduction effect was higher than in P deficiency or salt stress alone. This result is in contrast to those of other researchers (Abbas et al., 2018; Talbi Zribi et al., 2011; Tang et al., 2019), who respectively found that growth responses of barley, wheat and maize plants to combined P and salt stress, and to P deficiency alone were similar. In those studies, the authors demonstrated that the addition effect of salt to P deficiency did not further reduce plant growth. The opposite results observed in the present study could be related to the high concentration of salt (EC = 12 ds m^{-1}) used. In fact, in above mentioned previous studies, the salt concentration used was lower (EC = 8-10 ds m^{-1}). Therefore, as moderately salt tolerant plants, barley, wheat and maize are capable to withstand certain concentrations of salt. However, Phang and colleagues suggested that the outcome of combined effects of two constraints on plants growth might depend to the type of the stress imposed, the plant species and even the cultivars (Phang et al., 2009).

In our study growth of shoots and roots of maize cultivars exhibited different responses to P deficiency and salinity. Shoot growth of maize plants was reduced more markedly under P deficiency than under salt stress. It has been frequently suggested that shoots are organs that are affected more by nutrient deficiency (e.g., deficiency of N, P, and Fe) than by salinity (Talbi Zribi et al., 2015; Tang et al., 2019). The significant increase in root length of all maize cultivars, observed under P stress alone is in line with the findings of (Wissuwa et al., 2005) who found that P deficiency stimulated root elongation in rice. Many physiological mechanisms related to P deficiency tolerance of crop plants have been reported by scientists before. Among described mechanisms, increase in root hair length and density, which would improve P uptake through an expansion of root surface area at minimal cost, is one adaptation to P deficiency commonly observed in most species (Aziz et al., 2013; Wissuwa et al., 2005).

As expected, the concentrations of Na^+ in all maize cultivars significantly increased under salt stress treatments, probably contributing to the reduction of plant growth observed for salt stressed plants. Na^+ is the primary cause of ion stress for many plants, which not only competitively inhibits K^+, Ca^{2+}, and Mg^{2+} uptake thus disturbing the intracellular ion balance but also interferes with N and P acquisition and utilization (Pan et al., 2019). The osmotic effect is the first response to salinity and it reduces growth, mainly due to water deficiency in the plant tissues. The ionic effect is characterized by excessive accumulation of toxic ions in the cells, which slows growth by interfering with some important biochemical processes (Flowers and Colmer, 2015). Previous studies reported a significant increase of Na^+ due to salinity in many plants including maize, wheat, barley and white clover (Abbas et al., 2018; Farooq et al., 2015; Talbi Zribi et al., 2011). In our study, we found for most cases that Na^+ concentrations in combined P and salt stress were higher than in salt stress alone in all maize cultivars. These results did not agree with former studies that found Na^+ concentrations in barley and wheat plants were significantly lower under combined P and salt than in salt stress alone (Abbas et al., 2018; Talbi Zribi et al., 2011).

K^+ concentrations and K^+/Na^+ ratios in shoots and roots of all maize plants were decreased under all applied stress conditions (salinity, P deficiency, and combined P and salt stress) as compared to control plants growing under normal condition. Similar results were found by Abbas, when investigating the responses of two wheat cultivars "Jandorai" and "Janz" to the same growth conditions (Abbas et al., 2018). Decreased K^+ uptake due to P deficiency has already been reported (Saleque et al., 2001). It is well known that K^+ plays an important role in salt tolerance in plants (Farooq et al., 2015) and competition between K^+ and Na^+ under salt stress severely reduces K^+ concentration in both leaves and roots of maize. An imbalance in K^+ concentration can lead to severe water deficit due to a malfunctioning of guard cells of the stomata, as they are controlled by movements of K^+, and stomatal regulation is the major mechanism controlling the water balance of higher plants (Cordero et al., 2012).

P deficiency and combined P and salt stress treatments caused significant decreases in shoot and root P concentrations in all maize cultivars, as also noticed by (Fahad et al., 2016) in rice. Surprisingly, salinity did not significantly affect the shoot and root concentrations in all maize cultivars. We observed a slide increase or decrease in P concentrations due to salinity depending on the cultivar and the plant organ. (Silberbush and Ben-Asher, 1989) found that NaCl did not affect P uptake of peanut plants, as observed in different maize cultivars, in the present study. However, controversial outcome about the effect of salinity on P uptake were also reported in the literature. Studies of (Kaya et al., 2001) and (Abbas et al., 2018) respectively, on lettuce and wheat, showed that salt stress reduced P concentrations in all plant tissues, while (Phang et al., 2009) found that shoot and root P concentrations increased in barley grass and cultivated barley genotypes in response to salinity. Thus, (Grattan and Grieve, 1998) suggested that the interaction between salinity and P nutrition of plants depends to a large extent on the plant species (or cultivar), physiological developmental stage, environment, salt concentration, and P availability in the substrate.

Plant-bacteria associations can occur at different levels, and the genetic characteristics of both the bacteria and host plant can define the result of inoculation response in a given association (Azevedo et al., 2005). Bacteria with phosphate solubilizing and salt tolerance abilities, including *Arthrobacter* and *Bacillus* species have been successfully employed to improve growth of peanut (Jiang et al., 2018) and tomato (Tchuisseu et al.2019, submitted) plants in recent studies. However, our present study demonstrated for the first time that growth of maize plants exposed to combined P and salt stress was improved by bacterial inoculation. In general, we found that the compensation of reduce growth of maize cultivars observed in the present study under combined P and salt stress was significantly enhanced by *Arthrobacter* and *Bacillus* inoculations. The significant increases in plant growth with bacterial inoculations under combined P and salt stress match the findings of (Jiang et al., 2018).

Our results revealed that the bacterial inoculation effect was maize cultivar and bacterial strain dependent. Significant differential responses of maize cultivars to bacterial inoculations in greenhouse experiment were observed and the composite cultivar CMS 8704 which was most affected by the combined P and salt stress, responded best to bacterial inoculation. (Alam et al., 2003) found also a cultivar variation in the stimulation of rice growth inoculated with plant growth-promoting bacteria. Differences between responses of maize cultivars to bacterial inoculation may result from their genotypic variations. Although maize cultivars are botanically similar, they have a wide range of variation in seed size and origin (parent source) (Imran et al., 2015). It seems that the extent of bacterial effects on plant growth varied with the species or variety of the host plant as demonstrated in our work. Identification of model cultivar with positive and negative responses is thus useful for comparative studies involving association with PGPR for quantification of the responses under different environmental conditions

(Neiverth et al., 2014). Bacterial strains exhibited also significantly different effects on maize plant growth. For example there was a high positive bacterial - cultivar affinity between *Bacillu*s strain V62 and maize cultivar CMS 8704 in the present study. This bacterial strain exhibited the highest efficiency in increasing all the measured growth parameters in composite cultivar CMS 8704, while in other cultivars its efficiency varied with the parameter. Differences in growth promotion were also described for a *Bacillus* sp. in two lima bean varieties (Berger et al., 2018; Lima et al., 2016). The variability between the effects of the single bacterial strains on maize plant growth as observed in this study could be related to their individual plant growth – promoting (PGP) capabilities. The bacterial strains have previously been characterized and selected for PGP traits and were reported to have different abilities. Some strains, mainly *Arthrobacter* strains V54 and 64, and *Bacillus* strains V39 and V62 were found to possess more PGP traits and to be more efficient as compared to V84 and V1 with less traits (Tchuisseu Tchakounté et al., 2018). Likewise, our results showed that those strains comprising higher PGP trait numbers induced a higher and more stable effect *in vivo* with the different maize cultivars.

Combined P and salt stress increased Na^+ and decreased K^+ concentration, lowering therefore the K^+/Na^+ ratio in non-inoculated control plants. Decreasing Na^+ and increasing K^+ levels, resulting in higher K^+/Na^+ ratio is a strategy used by plants for alleviating salt-induced stress (Pan et al., 2019). In line with these findings, plants inoculated with *Arthrobacter* and *Bacillus* strains had lower shoot Na^+ concentrations than the non-inoculated control plants in all maize cultivars. In addition, except for *Bacillus* strain V1 in root K^+/Na^+ ratio, all bacterial strains resulted in increased K^+ content and K^+/Na^+ ratio in CMS 8704, as compared to their respective non-inoculated control plants. Results of (Rojas-Tapias et al., 2012) showed that inoculation with two *Azotobacter* strains helped maize plant cope with saline condition by decreasing Na^+ and increasing K^+. *Bacillus s*train GB03 was also reported to significantly decrease Na^+ accumulation and increase K^+/Na^+ in both shoots and roots of white clover under salt stress (Han et al., 2014). The lower Na^+ uptake and higher K^+ uptake and consequently the higher K^+/Na^+ ratio, in the PGPR-inoculated plants compared to control plant support the fact that K^+ levels are important for the osmotic potential in the vacuoles that helps to maintain high tissue water content. The capacity of inoculated plants to maintain high levels of K^+ during combined salt + P stress could be crucial for maintaining their hydration and turgidity (Cordero et al., 2012).

In the current study, increasing P uptake in maize plant by *Arthrobacter* and *Bacillus* inoculation was found in all maize cultivars except in composite CMS 8501. *Bacillus* strain V39 exhibited the significant most stable effect and showed an increase of up to 154.5%, 32.5% and 45.5%, respectively in CMS 8704, CHH 101 and CHH 103. A recent study reported that *Bacillus subtilis* increased P uptake by 40% in cucumber plant (Ana María and Delgado,

2016). The increase in plants P uptake observed in inoculated plants, as well as growth promotion of different maize varieties may suggests that bacterial strains exert their beneficial effect in the plant growth substrate by solubilizing the hardly available phosphate source applied under saline condition. This supports the mechanism that inoculated bacterial strains could decrease toxic ion acquisition and maintain the intracellular ionic equilibrium and increase nutrient availability to plants (Pan et al., 2019). However, we also observed that despite several bacterial strains enhanced plant growth of composite cultivar CMS 8501, none of them significantly increased P uptake in this cultivar. Similarly, (de Freitas et al., 1997) found that phosphate solubilizing bacteria (PSB) belonging to *Bacillus sp.* and *Xanthomonas maltophilia* were able to promote growth of canola plants without enhancing plant P uptake in previous studies, implying that these bacteria can influence plant growth by means other than phosphate solubilization. Another mechanism of plant growth stimulation under Combined P and salt stress could be related to bacterial phytohormone production abilities. The stimulation of plant growth and nutrient acquisition by beneficial rhizobacteria has been correlated to the biosynthesis of plant growth regulators, including gibberellins (Khan et al., 2014), auxins (Pereira et al., 2016), cytokinins (Kudoyarova et al., 2014) and ABA (Sgroy et al., 2009).

In addition to their preceding plant growth-promoting traits, the findings of the present study show the capacity of our selected bacterial strains to produce varied amounts of IAA, ABA, GA and cytokinins. Phytohormones are among the most important plant growth regulators, known for having a prominent impact on plant metabolism, and additionally, they play a vital role in the stimulation of plant defense response mechanisms against stresses (Egamberdieva et al., 2017b). The production Of IAA by *Arthrobacter* and *Bacillus* strains as observed in our study is consistent with the findings of a previous study reporting that *Arthrobacter* and *Bacillus* strains isolated from maize and wheat rhizospheres were able to produce IAA (Upadhyay et al., 2009; Zahid et al., 2015). IAA-producing microorganisms increase the root growth and root length of plants that enables the plant to obtain more nutrients from the soil and have the potential to positively regulate salt stress tolerance of plants (Jiang et al., 2018). Additionally, the ability of bacterial strains to produce ABA is also crucial for the plant growth under stress condition. It has been previously reported that some bacterial species that interact with plants or live in the soil, synthesize ABA (Shahzad et al., 2017). ABA plays an essential part in acting toward varied range of stresses like heavy metal stress, drought, thermal or heat stress, high level of salinity, low temperature, and radiation stress. ABA accumulation in roots and shoots mitigate the various inhibitory effects on growth, photosynthesis and assimilate translocation. The relationship between salt tolerance and ABA production is due to the accumulation of compatible solutes like sugars and proline in root vacuoles, as well as Ca^{2+} and K^{+}, which neutralize the effect of Na^{+} and Cl^{-} (Numan et al., 2018). The capacity to produce cytokinin, as observed in our *Arthrobacter* and *Bacillus* strains was also found in the study of (Irum et al.,

2009), stating that cytokinin-producing species, such as *Arthrobacter*, *Bacillus*, *Azospirillum*, and *Pseudomonas*, were able to stimulate the root development of plants. Cytokinins are involved in the maintaining of cellular proliferation and differentiation and the prevention of senescence, therefore leading to the inhibition of premature leaf senescence.

In a recent study, *Bacillus amyloliquefaciens* associated with rice (*Oryza sativa* L.) synthesized varied amount of gibberellins such as, GA20, GA36, GA24, GA4, GA53, GA5 and GA8 (Shahzad et al., 2016). Likewise, we observed that the different bacterial strains produced various gibberellins (GA1, GA3, GA4, GA5, GA7, GA8, GA9, GA13, GA15, GA19, GA20, GA24, GA29, GA34, GA44, GA51 and GA53). Although it has been demonstrated that *Arthrobacter* are able to produce GA (Upadhyay et al., 2009) this is the first report to show a wide range of GAs produced by this genus.

Maize requires a high demand of N for its growth. Thus, *Arthrobacter* strain V64 and *Bacillus* strain V39 with the potential to fix atmospheric nitrogen (besides having other PGP traits) were expected to exhibit the best performance on maize growth in greenhouse. However, our results showed that the highest growth enhancement was found in *Bacillus* strain V62. Though this strain did not possess the ability to fix atmospheric N_2, it was able to solubilize seven different phosphate sources even under salt stress condition and produced high amount of gibberellins mainly GA1, GA4, GA7 and GA24. Performing phosphate solubilization and gibberellin production seem to add a better growth promoting effect than N_2 fixation. This observation is in line with the findings of a recent study suggesting that *Bacillus amyloliquefaciens* H-2-5 promote Chinese cabbage, radish, tomato and mustard plants growth due to its gibberellin (GA4, GA8, GA9, GA19, and GA20) production and phosphate solubilization abilities (Kim et al., 2017). To the best of our knowledge, the present study is the first to report the contribution of *Arthrobacter sp. and Bacillus sp.* to the growth of maize crops under combined salt + P stress through the solubilization of phosphate and the production of phytohormones. In general, the result of the present study rebut our hypothesis that bacterial growth-promoting effects will be higher in hybrids than composites cultivars, implying that further work is crucial for understanding such specific plant-bacteria interactions.

5.6. Conclusion

In summary, we demonstrated based on our *in- vitro* and greenhouse studies that *Arthrobacter* and *Bacillus* strains could reduce the combined detrimental effects of salinity and P deficiency on the growth of different maize cultivars, and this dependent upon both the maize cultivar and the bacterial strain. The present results point out that bacterial amelioration of combined P and salt stress could be the integration of several aspects including improving plant mineral nutrition, and promoting plant growth by bacterial synthesis of plant hormones. Additionally,

our study suggests selecting maize cultivars that are positively responding to inoculation with indigenous bacteria in P-deficient and salt-affected soils and supports the fact that co-selecting a proper maize cultivar along with a highly efficient and appropriate PGPR strain is necessary for improving plant growth under a given condition. This is an important step for moving the agricultural practices towards sustainability. Further work should focus on developing formulations of the selected bacterial strains for their application in the field.

CHAPTER 6: SYNTHESIS

6.1. General discussion

Nutrient deficiencies in soil, mainly in phosphorus (P) and nitrogen (N), coupled to salinity and the impoverishment of agricultural soils, are a severe problem for agricultural production worldwide. This thesis contributes with an environmental-friendly alternative approach that could be exploited to improve maize and tomato plant growth in P-deficient and salt-affected soils.

The principal findings of our research were: i) the rhizosphere of maize grown in Cameroon host a high diversity of cultivable bacteria exhibiting multiple plant growth-promoting traits among which, phosphate solubilization, atmospheric nitrogen fixation, phytohormones and siderophores production activities and salinity tolerance and motility abilities. ii) Bio-inoculation with selected bacterial strains from the most abundant genera of the maize rhizosphere community (*Bacillus* and *Arthrobacter*, respectively) represents an ecofriendly alternative to improve maize growth *in vitro* under normal condition and *in vivo* in greenhouse under combined P and salt stress condition; iii) Growth promoting-effect of selected bacterial strains is not specific to the native plant, since they were also able to enhance tomato plant growth under combined P and salt stress condition; iv) Multifaceted bacteria with more plant growth-promoting traits showed a better effect in improving maize and tomato plants growth under combined P and salt stress. V) Co-selection of efficient bacterial strain and maize cultivar enhanced the effectiveness of bacterial inoculation.

In the present thesis, we first focused on the isolation and identification of bacteria based on their 16S rRNA gene sequencing and phylogenetic affiliation and analyzed quantitatively the specific group obtained to find out their distribution in the studied site and get an overview of the community structure of cultivable bacteria associated with maize grown in Cameroon. Then, we *in vitro* characterized all the isolated bacteria for different plant growth-promoting traits using biochemical test in order to analyze the distribution of these different PGP traits between the different bacterial groups (chapter 3). There are two key findings of this part of our study that are worth to be highlighted: 1) isolates belonging to *Bacillus* and *Arthrobacter genera* were the most abundant in the bacterial community associated with maize grown in Cameroon; 2) the newly selected bacterial strains significantly increased hypocotyl and root length of maize seedlings *in vitro*. The enrichment of members from the genera *Arthrobacter* and *Bacillus* in maize rhizosphere in the studied site, suggests that future efforts should be concentrated in isolating these bacteria in other regions of the country. In addition, this dominance could be explained by the high adaptation of these two genera to the specific environmental conditions. Therefore, six bacterial, three *Arthrobacter* (V54, V64 and V84) and three *Bacillus* (V1, V39 and V62) strains were selected for further studies. The high abundance

of member of *Firmicutes* and *Actinobacteria* phyla and less dominance of members of *Proteabacteria,* as observed in our study did not agree with study of Arruda et al. (2014) reporting that *Proteobacteria* is the most common of the dominant populations found in the rhizosphere of different plant species. *Proteobacteria* was also found to be the prevailing group by several studies while investigating the bacterial communities from the rhizosphere of maize plants (Roesch et al., 2008; Chelius and Triplet, 2001). Besides the soil type, plant species, plant cultivar, plant stage, microbial interaction and farming practices known as the common factors affecting the bacterial community in the rhizosphere (Lopez-Reyes et al., 2015; Cavaglieri et al., 2007), the study technique used seems to also have an impact of the bacterial communities associated with maize. The dominance of certain bacterial group appears to be related either to the culture- dependent or independent methods. Indeed, in the same study Pereira et al. (2011) analyzed the bacterial diversity associated with maize through culture-dependent and culture-independent methods and found that *Firmicutes,* mainly *Bacillus* were the predominant group in the culture –dependent method, while the culture independent method revealed that *Proteobacteria,* especially *Enterobacter*, *Pseudomonas*, *Erwinia* were the most abundant in the community.

The results presented in this thesis based on the culture-dependent approach are in consistence with the aim of this study; to identify microbes for agricultural applications. Our approach using in silico selection procedures (using a combination of molecular / bioinformatics tools and *in vitro* studies) provides insight into the community structure and the functional differences between the different bacterial taxonomic groups and represent a time saving and cost efficient method to detect effective PGP bacteria. Nevertheless some limitations are worthy to be mentioned. On one hand, the identification of bacteria based on the 16S rRNA gene sequencing was complicated due to numerous ambiguous results: 1) in some cases, different results were obtained when the same sequence was compared against different databases. For example, isolate V17 using blast search resulted in the highest matches to *Bacillus* genus in NCBI database, while in RDP V17 was closest to *Planococcaceae* family. 2) 16S rRNA gene sequencing was not enough to differentiate the isolate at the specie or even genus level. Phylogenetic analysis based on the 16S rRNA gene sequence could not differentiate between strains belonging to *Bacillus cereus* and *Bacillus thuringiensis* in the *Firmicutes* group nor *Arthrobacter* and *Sinomonas* strains in the Actinobacteria group. The failure of 16S rRNA gene sequencing in bacterial identification as observed in our study may be related to the use of the partial sequence instead of the full length 16S rRNA gene sequence. However, recent studies reported difficulties to identify *Bacillus* species using the full length 16S rRNA gene sequence. This could be explained by the fact that two distinct species, including species of *Bacillus* may possess identical 16S rRNA gene sequences as the 16S rRNA gene is known to be highly conserved and determination of species and strain

distinctions relies upon the resolution of only small differences between sequences. Moreover, reliance on a single molecular method for species identification, such as 16S rRNA gene, cannot take into account small evolutionary changes, such as point mutations (Stackebrandt et al. 2002). Since, the efficiency of 16S rRNA gene sequencing is shown to be limited for the identification of bacteria as observed in the present study, we concluded from our analyses that identification of bacteria based on the 16S rRNA gene sequencing requires additional tests. Thus, in practice, a polyphasic approach including alternate gene targets performed in parallel with the examination of number phenotypic properties is necessary for definitive species identification. On the other hand, although isolation of cultivable bacteria is appropriate for functional analysis, this approach often shows a rather limited diversity because a high percentage of naturally occurring bacteria remains in a not yet cultivable state (David Galeote et al.; 2017). A full understanding of microbial community of maize requires the complementation of this work with culture independent approaches that uses advanced genomic and bioinformatics tools such Illumina pyrosequencing, DNA/RNA microarrays and molecular fingerprinting techniques (David Galeote et al., 2017; Mutai et al., 2017). Culture independent molecular methods provide additional information on the diversity of bacterial communities by indicating the presence of innumerous not yet cultivated species and have been using recently to explore the microbiota from several plant species, such as the model plant species Arabidopsis (Schlaeppi et al., 2014), and economically important crop plants such as maize (Peiffer et al., 2013) and tomato (Tian et al., 2015). However, Shade et al. (2012) reported that culture-independent based techniques capture fewer members of low abundance organisms, the so called rare biosphere than the culture based approach. Therefore, a multimethodological approach using conventional techniques such as bacterial isolation and physiological studies, together with molecular analysis, will be necessary to get a comprehensive view of the microbial community in different environments.

Bacteria isolated from maize rhizosphere exhibited multiple PGP traits among which phosphate solubilization. The first selection of isolated bacteria was done based on their ability to solubilize seven different phosphate sources on plates and we assumed that the efficiency of a strain will be related to its capacity to so solubilize a wide range of mineral phosphate sources. In a recent study, Kumar and co-workers (2014) assessed the efficiency of 12 *Bacillus* strains for their phosphate solubilizing efficiency on plates using several inorganic phosphate sources. As the direct measurement of phosphate solubilization in broth assay is likely to give more reliable results than a regular plate assay, the screened the six selected bacterial strains were further tested for their ability to solubilize tricalcium phosphate (TCP) and Cameroonian rock phosphate (CRP) in NBRIP broth. The results showed that all the six tested *Arthrobacter* and *Bacillus* strains were able to mobilize TCP and CRP in shaking culture after 5 days. It is generally, accepted that the major mechanism of mineral phosphate solubilization is through

the action of organic acids produced by soil microorganisms (Alori et al., 2017). Among the organic acids, gluconic acid seems to be the most frequent agent of phosphate solubilizing bacteria and it is reported as the principal organic acid produced by phosphate solubilizing bacteria. *pqqC* gene is one of the gene involved in the production of gluconic acid. In order to define the P solubilization pathway of the six selected *Arthrobacter* and *Bacillus* strains, amplification of *pqqC* gene using a universal primer as previously proposed by Anzuay et al. (2013) was conducted. Based on our analysis, the *pqqC* gene was detected in all *Arthrobacter* strains, while in *Bacillus* strains, this gene was not present. According to our results, we hypothesized that the selected *Arthrobacter* and *Bacillus* strains may have different mechanisms in solubilizing mineral P. However, this hypothesis should be approached experimentally. For instance, determining the organic acid produced by strains in pure culture and sequencing their whole genome and defining the genes involved in P solubilization will give us insights in the P solubilizing pathways of the respective strains.

Stressful conditions prevailing in the soil are known to have direct effect on the activity, survival and proliferation of the soil microbiota (Kumar et al., 2014). In Cameroon P-deficient and salt-affected soils are very common. Thus, selected *Arthrobacter* and *Bacillus* strains should be able to exhibit their growth- promoting abilities under such stressful conditions. In order to confirm that isolated strain will be really be efficient in Cameroon low soil fertility (P deficiency and salinity, for instance), the six selected strains were further tested for their efficacy to solubilize tricalcium phosphate and Cameroonian rock phosphate in shaking culture under saline condition (Chapter 4). We expected that salinity would negatively influence the efficiency of bacterial strains. However, this was not the case for all strains, mainly for *Bacillus* strain V39. We concluded from our results that the P solubilization capacity varied with the strain, the phosphate source, and the growth condition.

The importance of selected *Arthrobacter* and *Bacillus* was particularly significant regarding their beneficial effects in improving P nutrition under double P and salt stress condition through the solubilization of rock phosphate and thus, plant growth. These selected PGPR of maize were not plant specific plant-growth promoters, as besides stimulating the growth of maize, their host plant, they also enhanced the growth of tomato plant in greenhouse experiments under the same stress condition. Likewise, Fankem et al. (2014) reported maize growth-promotion by *Pseudomonas, Burkholderia* and *Enterobacter* strains isolated from the rhizosphere of *Elaeis guineensis*. Our results imply that the selected *Arthrobacter* and *Bacillus* strains could be used for several crops and may be of great agricultural interest due to their growth-promoting effect on maize and tomato. Nevertheless, further tests on other plant species are needed to confirm their universal plant promoting effect.

In general, our results showed that there was a huge significant variability in the efficiency of the six selected bacterial strains in promoting plant *in vivo*. In fact *Arthrobacter* (V54 and V64) and *Bacillus* (V39 and V62) strains with higher number of PGP traits in the tested range of activities in pure cultures induced greater growth effects in greenhouse experiments under double P and salt stress. This indicates that bacteria possessing more PGP attributes could be more effective PGPR to improve crop growth under multiple stresses conditions. Similarly, the inoculation of microorganisms with multiple PGP attributes proved to be more significant for increasing the growth and yield in recent studies (Etesami and Maheshwari, 2018; Hussein et al. 2013). The stimulated plant growth by these bacteria may be related the net result of the synergetic combination of several PGP activities such as: phosphate solubilization, N_2 fixation and phytohormones production. However, this hypothesis should be proven experimentally. For example, knocking out genes involved in these PGPR traits will allow comparing the performance as bio-inoculants of mutants versus wild type strains.

Also important besides selecting efficient PGPR for the development of inoculants for agriculture, is the choice of adapted cultivars that benefit from association with these bacteria. Indeed, the genetic interaction between strain and the plant cultivars has been acknowledged as a key to improving the level and reliability of plant growth stimulation by PGPR (Egamberdieva, 2010). Therefore, in the fifth chapter of the thesis we evaluated the response of four maize cultivars two composite (CMS 8704 and CMS 8501) and two hybrids (CHH 101 and CHH 103) to inoculation with the six selected *Arthrobacter* and *Bacillus* in greenhouse experiment under combined P and salt stress condition. The aim was to find the best positive bacterial strain- maize cultivar interaction for further improvement of plant growth promotion. Our results showed that, , although all the six bacterial strains positively affected the growth of all maize cultivars, bacterial plant growth stimulating effect was higher in composites cultivars mainly CMS 8704. Our results suggest that strain x variety interaction is as important as that of strain or crop variety alone crop improvement under a specific stress condition. Likewise, studies of Egamberdieva (2010) and Imran et al. (2015) have also demonstrated the crucial role played by the strain - cultivar interaction respectively in the enhancement of wheat and chickpea growth. The variability among the different maize cultivars to bacterial inoculation supports the fact that each host cultivar has a variable potential for response toward bacterial inoculation. We concluded from the results that the effectiveness of PGPR can be improved by using adapted plant cultivar under a specific stress condition and that the co-selection of all three main contributors, i.e., plant genotype, microbe and soil is indispensable for increasing PGPR application in agriculture.

6.2. General conclusion and significance of the study

The research conducted in this thesis has demonstrated the high abundance of cultivable bacteria associated with maize in Cameroon. The isolation, characterization and selection of a limited number of rhizobacterial strains have revealed that rhizosphere of maize grown in Cameroon harbor effective PGPR strains that can be exploited for further biotechnological applications. In addition, our results highlight the capacity of the selected bacterial strains, members of to the most abundant genera (*Arthrobacter* and *Bacillus*) found in the community, to solubilize phosphate in the presence of high salt concentrations and to promote maize and tomato plants growth under combined P and salt stress conditions. We also found that the bacterial amelioration of combined P and salt stress could be the integration of several aspects including improving plant mineral nutrition, and promoting plant growth by bacterial synthesis of plant hormones. Furthermore, our study assists the selection of maize cultivars more responsive to inoculation with indigenous bacteria in P- deficient and salt- affected soils, which represents an additional step to improve the bacteria efficiency under a given condition. Prior to our studies, the role of native bacteria from Cameroon soil as potential bio-inoculants for maize and tomato has not been assessed as a sustainable alternative to improve plant growth in P-deficient and salt- affected soils. At the end of this thesis we are able to suggest that bio-inoculation with native PGPR should be considered as an eco-friendly strategy to enhance the growth of plants in greenhouse conditions.

This doctoral thesis represents the beginning of a future project that aims at the development of a native bacterial inoculant to improve plant growth in low-fertility soils in Cameroon and the results obtained from this work have significant values:

- Like in many sub-Sahara African countries, studies on plant growth-promoting rhizobacteria (PGPR) are still largely unexploited in Cameroon. The data of this thesis provides the first collection of bacteria associated with maize in Cameroon and therefore is valuable for further studies of microbial communities associated with plants in the country.
- The different PGP traits tested give insight into the functional difference between bacterial groups found in the maize rhizosphere and can be useful to understand the ecological role of newly isolated bacteria in their specific environment.
- The knowledge about the bacterial groups and their functional traits may contribute to improve management practices regarding plant resistance to plant nutrition (mainly P nutrition) and salinity.
- The data of the present thesis may help in promoting microbial bio-fertilizers based on native rhizobacteria in sub-Saharan Africa, especially in Cameroon and enhancing sustainable crop production systems.

6.3. Outlook

As mentioned in the general conclusion, this doctoral thesis represents the beginning of a future project that aims at the development of a native bacterial inoculant to improve plant growth in low-fertility soils in Cameroon. Therefore, for further work, the role of *selected Arthrobacter and Baciilus* strains as bio-inoculant, single or in consortium should be investigated in field trials in order to confirm the promising growth-promoting effect observed in greenhouse conditions. This could be possible with the collaboration of local farmers in Cameroon and we are optimistic with the idea that this can be approached in the near future.

On the other hand, the whole genome sequence of selected strains could allow performing fundamental research to elucidate the mechanisms used by these to contribute to plant growth and P nutrition under combined P and salt stress. For instance, by searching for genes responsible for phosphate solubilization and salinity tolerance in the different strains will help to compare the pathway used by each strain during the phosphate solubilization and salinity tolerance mechanisms. Another interesting approach will be to analyze the performance under the same stress condition of mutant strains knocked out in genes responsible for some of the PGP traits of these bacterial strains to evaluate if one of them is critical for exerting their plant growth-promoting mechanisms. And finally, it will be of high interest to *in silico* prove the phosphate solubilization and salinity tolerance mechanisms in model experiments using bacterial transcriptome analysis and the quantification of marker gene expression.

Appendices

Table 15: Different bacterial morphotypes (see chapter 3.4.1) isolated from maize rhizosphere soil and their relative abundance

Morpho-types	Replication 1				Replication 2				Replication 3				Replication 4				Average
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{8} CFU
SB1		1									2			1			0.0055
SB2		2															0.005
SB3											1			1			0.00275
SB4					2			1			1						0.050275
SB5											1						0.00025
SB6											28		1				0.032
SB7		5			5				2					7			0.205
SB8		4			2				1								0.085
SB9									5								0.125
SB10														1			0.0025
SB11					1												0.025
SB12		1															0.0025
SB13		1									2						0.003
SB14		4				3								2			0.0225
SB15		4									1						0.01025
SB26		1										1		2			0.007525
SB17											1		2	1			0.05275
SB18											4				2		0.0015
SB19							1				1						0.0015
SB20															3		0.00075
SB21															8		0.002
SB22						2								3			0.0125
SB23														1			0.0025
SB24												5					0.000125
SB25												1					0.000025
SB26											1	1					0.000275
SB27				30								7					0.000925
SB28											1	1					0.000275
SB29												1					0.000025
SB30				12							4			1		1	0.003825
SB31																47	0.001175
Total		23	0	42	10	5	1	1	8	0	48	17	3	20	13	48	0.6642

CFU= colony forming unit

Table 16: Occurrence and characterization of bacterial strains to tolerate different concentration of salt at genus, family and phylum level

	Characterization level	Number of isolates	2% NaCl	4% NaCl	6% NaCl	8% NaCl
Genera	***Aerococcus***	1	1	1	1	1
	Amycolatopsis	2	1	1	1	0
	Arthrobacter	25	24	15	5	1
	Bacillus	45	38	31	22	10
	Burkholderia	3	2	0	0	0
	Domibacillus	1	0	0	0	0
	Kitasatospora	1	0	0	0	0
	Leifsonia	8	7	1	0	0
	Lysinibacillus	3	3	2	1	0
	Microbacterium	1	1	0	0	0
	Micrococcus	4	4	4	4	2
	Mycobacterium	1	1	1	0	0
	Paenibacillus	7	7	4	1	1
	Roseomonas	1	0	0	0	0
	Sinomonas	19	19	18	1	0
	Solibacillus	4	4	4	1	0
	Staphylococcus	11	11	8	8	8
	Streptomyces	3	1	1	1	1
	Unclassified Intrasporangiaceae	2	1	0	0	0
	Unclassified Planococcaceae	1	1	1	1	0
Total	**20**					
Families	***Acetobacteraceae***	1	0	0	0	0
	Aerococcaceae	1	1	1	1	1
	Bacillaceae	49	41	33	23	10
	Burkholderiaceae	3	2	0	0	0
	Intrasporangiaceae	2	1	0	0	0
	Microbacteriaceae	9	8	1	0	0
	Micrococcaceae	48	47	37	10	3
	Mycobacteriaceae	1	1	1	0	0
	Paenibacillaceae	7	7	4	1	1
	Planococcaceae	5	5	5	2	0
	Pseudonocardiaceae	2	1	1	1	0
	Staphylococcaceae	11	11	8	8	8
	Streptomycetaceae	4	1	1	1	1
Total	**13**					
Phyla	***Actinobacteria***	66	59	41	12	4
	Firmicutes	73	65	51	35	20
	Proteobacteria	4	2	0	0	0
Total	**3**	**143**	**126**	**92**	**47**	**24**

Table 17: Occurrence and characterization of bacterial strains to solubilize different types of inorganic phosphate source at genus, family and phylum level

	Characterization level	Number of isolates	Chemical inorganic phosphate		Rock phosphate (RP)					Total P solubilizing isolates
			Tricalcium Phosphate	Hydroxyapatite	Malian RP	Cameroonian RP	Algerian RP	Mexican RP	Moroccan RP	
Genera	*Aerococcus*	1	0	0	0	0	0	0	0	0
	Amycolatopsis	2	1	1	1	0	0	1	0	1
	Arthrobacter	25	19	14	18	10	15	12	2	19
	Bacillus	45	18	14	17	13	11	12	2	20
	Burkholderia	3	0	0	0	0	0	0	0	0
	Domibacillus	1	0	0	0	0	0	0	0	0
	Kitasatospora	1	0	0	0	1	0	0	0	1
	Leifsonia	8	3	2	3	2	1	1	0	3
	Lysinibacillus	3	0	0	0	0	0	0	0	0
	Microbacterium	1	1	1	1	1	1	1	0	1
	Micrococcus	4	3	2	3	3	2	2	0	3
	Mycobacterium	1	0	0	0	0	0	0	0	0
	Paenibacillus	7	5	5	3	2	4	4	0	7
	Roseomonas	1	0	0	1	1	0	0	0	1
	Sinomonas	19	12	1	11	6	3	2	0	12
	Solibacillus	4	0	0	0	0	0	0	0	0
	Staphylococcus	11	3	1	3	2	1	1	0	3
	Streptomyces	3	1	1	1	1	1	0	0	1
	Unclassified Intrasporangiaceae	2	0	0	0	0	0	0	0	0
	Unclassified Planococcaceae	1	0	0	0	0	0	0	0	0
Total	**20**									
Families	*Acetobacteraceae*	1	0	0	1	1	0	0	0	1
	Aerococcaceae	1	0	0	0	0	0	0	0	0
	Bacillaceae	49	18	14	17	13	11	12	2	20
	Burkholderiaceae	3	0	0	0	0	0	0	0	0
	Intrasporangiaceae	2	0	0	0	0	0	0	0	0
	Microbacteriaceae	9	4	3	4	3	2	2	0	4
	Micrococcaceae	48	34	17	32	19	20	16	2	34
	Mycobacteriaceae	1	0	0	0	0	0	0	0	0
	Paenibacillaceae	7	5	5	3	2	4	4	0	7
	Planococcaceae	5	0	0	0	0	0	0	0	0
	Pseudonocardiaceae	2	1	1	1	0	0	1	0	1
	Staphylococcaceae	11	3	1	3	2	1	1	0	3
	Streptomycetaceae	4	1	1	1	2	1	0	0	2
Total	**13**									
Phyla	*Actinobacteria*	66	40	22	38	24	23	19	2	41
	Firmicutes	73	26	20	23	17	16	17	2	30
	Proteobacteria	4	0	0	1	1	0	0	0	1
Total	**3**	**143**	**66**	**42**	**62**	**42**	**39**	**36**	**4**	**72**

Table 18: Occurrence and characterization of bacterial strains for *nifH* gene presence and siderophore production at genus, family and phylum level

	Characterization level	Number of isolates	*nifH* gene	Siderophore production
Genera	***Aerococcus***	1	0	0
	Amycolatopsis	2	0	0
	Arthrobacter	25	7	2
	Bacillus	45	3	13
	Burkholderia	3	0	1
	Domibacillus	1	0	0
	Kitasatospora	1	0	1
	Leifsonia	8	1	1
	Lysinibacillus	3	1	0
	Microbacterium	1	0	0
	Micrococcus	4	0	1
	Mycobacterium	1	0	1
	Paenibacillus	7	2	1
	Roseomonas	1	0	1
	Sinomonas	19	0	0
	Solibacillus	4	0	0
	Staphylococcus	11	1	5
	Streptomyces	3	0	0
	Unclassified Intrasporangiaceae	2	0	1
	Unclassified Planococcaceae	1	0	0
Total	**20**			
Families	***Acetobacteraceae***	1	0	1
	Aerococcaceae	1	0	0
	Bacillaceae	49	4	13
	Burkholderiaceae	3	0	1
	Intrasporangiaceae	2	0	1
	Microbacteriaceae	9	1	1
	Micrococcaceae	48	7	3
	Mycobacteriaceae	1	0	1
	Paenibacillaceae	7	2	1
	Planococcaceae	5	0	0
	Pseudonocardiaceae	2	0	0
	Staphylococcaceae	11	1	5
	Streptomycetaceae	4	0	1
Total	**13**			
Phyla	***Actinobacteria***	66	8	7
	Firmicutes	73	7	19
	Proteobacteria	4	0	2
Total	**3**	**143**	**15**	**28**

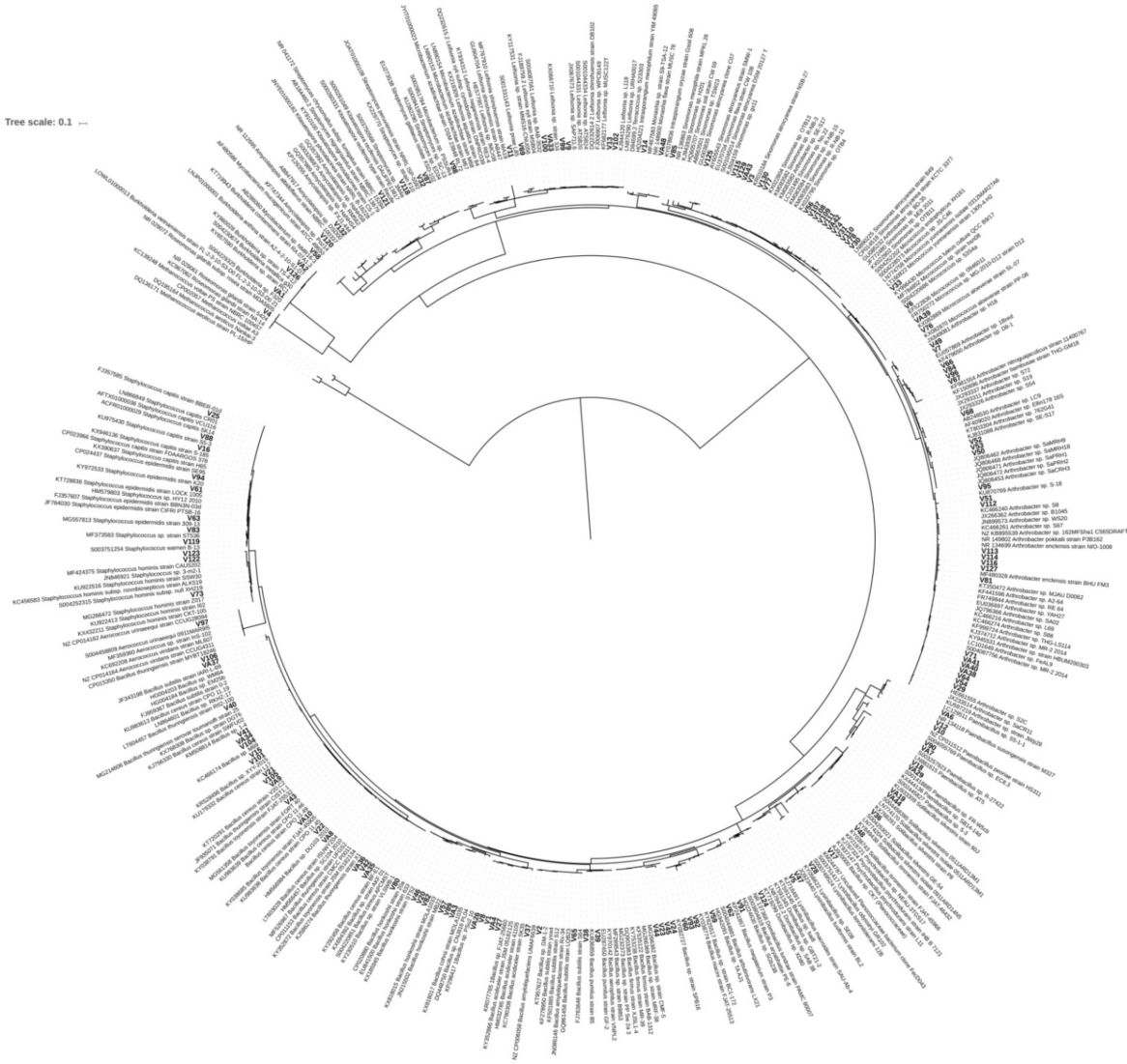


Figure 33. Phylogenetic tree based on 16S rDNA sequences revealing phylogenetic classification of the 143 isolates: the branch lengths displayed represent substitutions per site. The Maximum Likelihood tree was structured using the Tamura 3-parameter model and the neighbor joining method. *Methanococcus* ssp. was used as outgroup. Bootstrap support values are not shown here, but in Figure 7. The isolates between sequences are represented in bold.

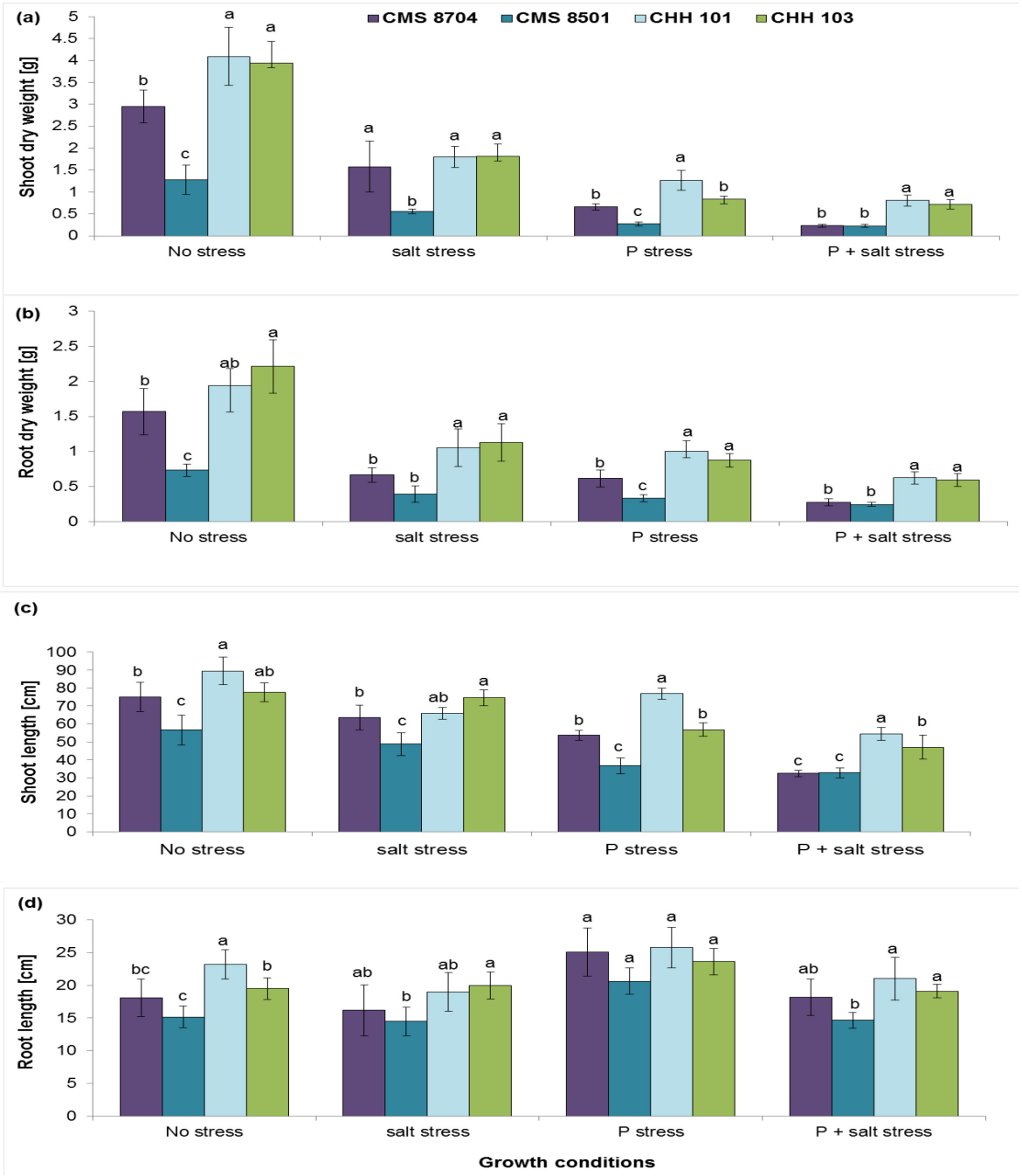


Figure 34. Growth response of maize cultivars to different growth conditions: shoot dry weight (a), root dry weight (b), shoot length (c) and root length (d) responses of two composites (CMS 8704 and CMS 8501) and two hybrids (CHH101 and CHH 103) maize cultivars to no stress, salt stress, P stress, and double P and salt stress conditions. Values are the means ± standard deviation of six replicates. For each cultivar, values sharing the same letter are not significantly different ($p \leq 0.05$) using the Tukey HSD test.

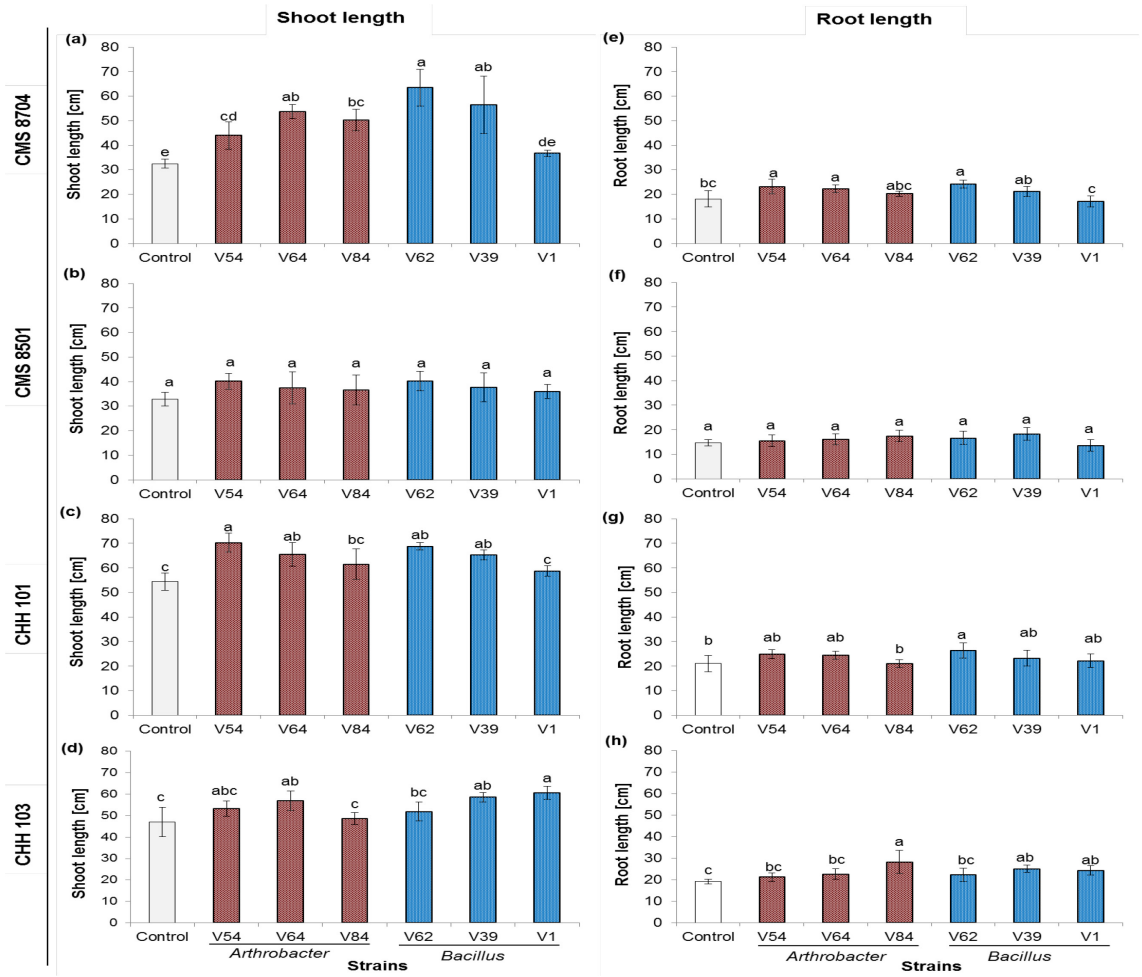


Figure 35. Bacterial inoculations promoted shoot and root of different cultivars under combined P and salt stress. Effects of *Arthrobacter* and *Bacillus* strains compared to the non-inoculated control on plant growth: (**a-d**) shoot and (**e-h**) root length, of different maize cultivars. Maize plants grown under combined P and salt were assessed six weeks after planting in a greenhouse. Values are means of six replications and error bars represent standard deviations. Different letters indicate significant differences between treatments within the same cultivar ($p < 0.05$) using the Tukey HSD test. P stress was applied using the hardly available Cameroonian rock phosphate and the salt stress on plants was induced by applying an electrical conductivity of 12 ds m^{-1}. Control (non-inoculated), V54, V64 and V84 (*Arthrobacter* sp.), V62, V39 and V1 (*Bacillus* sp)

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